# The defects underlying impaired T-cell immunity in chronic lymphocytic leukaemia: the impact of lenalidomide

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# Dedication

To John, Sylvia and Eleni.

# Acknowledgements

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# Statement of Work Undertaken

I performed all of the work described in this thesis with the following exceptions:

Collection of the CLL patient samples was done by Sameena Iqbal and the tissue bank staff.

Cytotoxicity assays, cell conjugation assays and confocal microscopy were performed by Alan Ramsay and Rewas Fatah.

Tracy Chaplin-Perkins performed the Affymetrix HGU133Plus2.0 Genechip hybridisations and initial analysis.

Ajanthah Sangaralingam performed the biostatistical analysis on the gene expression data.

Essam Ghazaly performed mass spectrometry to confirm the presence and absence of degradation of lenalidomide.

# Abstract

CLL T cells exhibit functional defects and alterations in gene expression that show similarities to exhausted T cells in chronic viral infections. It is unclear whether CLL T cells are truly exhausted, or whether these defects are restricted to expanded populations of CMV specific cells. The phenotype and function of T cells from CLL patients was compared to age- and CMV-serostatus-matched controls. There were an increased proportion of effector T cells in CLL patients and CMV-seropositive individuals. $CD8^+$ and $CD4^+$ T cells from CLL patients had increased expression of exhaustion markers CD160 and CD244 irrespective of CMV-serostatus, whereas increased PD1 expression on $CD8^+$ T cells was limited to CMV-seronegative patients. CLL $CD8^+$ T cells also showed defects in proliferation and cytotoxicity irrespective of CMV-serostatus, with the cytolytic defect caused by impaired granzyme packaging into vesicles and non-polarized degranulation. In contrast to virally-induced exhaustion, CLL T cells had increased expression of TBET and increased interferon-γ production, but normal IL-2 production.

As lenalidomide repairs the functional and phenotypic defects associated with T-cell exhaustion, the effect of this agent on the gene expression profiles of lymphocyte subsets from CLL patients and healthy controls was investigated. Lenalidomide induced the expression of genes involved in cytoskeletal signalling, lymphocyte activation, and proliferation. In particular, lenalidomide up-regulated the expression of several genes involved in tight junction signalling, a pathway that is potentially involved in lymphocyte motility, immune synapse formation, and transendothelial migration. This pathway was down-regulated in T cells from CLL patients, but, intriguingly, was up-regulated in CLL cells compared with healthy B cells. This pathway is known to be negatively regulated by a phosphatase, PP2A. Treatment of CLL cells and T cells with the PP2A inhibitor okadaic acid mimicked the effect of lenalidomide.

In conclusion, CLL T cells exhibit features of pseudo-exhaustion irrespective of CMV serostatus. Lenalidomide up-regulates tight junction signalling, which is down-regulated in CLL T cells. Inhibition of PP2A is implicated in the mechanism of action of lenalidomide on T cells.

# Publications

## Original articles

**Riches, J.C.,** Davies, J.K., McClanahan, F., Iqbal, S., Fatah, R., Agrawal, S., Ramsay, A.G., Gribben J.G. *T cells from CLL patient exhibit features of T-cell exhaustion but retain capacity for cytokine production* Blood 2013: 121(9):1612-21

Ene-Obong, A., Clear, A.J., Watt, J., Wang, J.,Fatah, R., **Riches, J.C.,** Marshall, J.F., Chin-Aleong, J., Chelala, C., Gribben, J.G., Ramsay, A.G., Kocher, H.M. *Activated Pancreatic Stellate Cells Sequester CD8+ T-Cells to Reduce Their Infiltration of the Juxtatumoral Compartment of Pancreatic Ductal Adenocarcinoma* Gastroenterology 2013 [In press]

## Review articles

Gribben, J.G., **Riches, J.C.** *Immunotherapeutic strategies including transplant: Eradication of Disease* Hematology Am Soc Hematol Educ Program 2013 [In press]

**Riches, J.C.,** Gribben, J.G. *Understanding the Immunodeficiency in Chronic Lymphocytic Leukemia: Potential Clinical Implications* Hematol Oncol Clin North Am. 2013 Apr:27(2):207-35

**Riches, J.C.,** Ramsay, A.G., Gribben, J.G. *Immune dysfunction in chronic lymphocytic leukemia: the role for immunotherapy*. Curr Pharm Des. 2012;18(23):3389-98

**Riches, J.C.,** Ramsay, A.G., Gribben, J.G. *Immune Reconstitution in Chronic Lymphocytic Leukemia.* Curr. Hematol. Malig. Rep. 2012 Mar;7(1):13-20.

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**Commentaries**

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**Riches, J.C.,** Gribben, J.G. *Interesting times in the diagnosis and treatment of CLL.* Oncology (Williston Park) 2009 Nov 15;23(12):1043-6

**Meeting abstracts**

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**Riches, J.C.,** Davies, J.K., McClanahan, F., Iqbal, S., Fatah, R., Agrawal, S., Ramsay, A.G., Gribben J.G. *Identification of an expanded population of terminally differentiated CD8+ T cells with a novel phenotypic and functional profile in patients with CLL*. Haematologica 2012; 97 (supplement 1) Abstract 1099

**Riches, J.C.,** Sangaralingam, A., Kiaii, S., Chaplin, T., Cekdemir, D., Iqbal, I., Ramsay, A.G., Gribben, J.G. *Impact Of Lenalidomide On Gene Expression Profiles Of Malignant And Immune Cells In Patients With Chronic Lymphocytic Leukemia*. Blood 2011; 118:21 Abstract 976

**Riches, J.C.,** Davies, J., Iqbal, S., Fatah, R., Agrawal, S., Ramsay, A.G., Gribben J.G. *T-cells from Patients With CLL Exhibit Phenotypic and Transcription Factor Profiles Of Exhaustion Independent of CMV Serostatus.* Blood 2011 118:21 Abstract 1780

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# List of Abbreviations

| | |
|---|---|
| ACDA | Acid citrate dextrose |
| ADCC | Antibody dependent cell-mediated cytotoxicity |
| AF | Alexa-Fluor |
| AICD | Activation induced cell death |
| AMPK | Adenosine monophosphate-activated protein kinase |
| AMPKK | Adenosine monophosphate-activated protein kinase kinase |
| AP1 | Activator protein 1 |
| APC | Antigen presenting cell |
| APC | Allophycocyanin |
| APRIL | A proliferation-inducing ligand |
| ARHGEF7 | Rho guanine nucleotide exchange factor (GEF) 7 |
| ARL11 | ADP-ribosylation factor-like 11 |
| Arp2/3 | Actin related proteins 2/3 |
| BAFF | B-cell activating factor |
| BATF | Basic leucine transcription factor ATF-like |
| BCL | B-cell lymphoma |
| BCR | B-cell receptor |
| BID | BCL-2 Interacting Domain |
| BLIMP1 | B-lymphocyte-induced maturation protein 1 |
| BLNK | B-cell linker protein |
| BMMC | Bone marrow mononuclear cell |
| BR | Bendamustine, rituximab |
| BSA | Bovine serum albumin |
| BTK | Bruton’s tyrosine kinase |
| CAR | Chimeric antigen receptor |
| CCL | C-C motif ligand |
| CCR | C-C motif receptor |
| CD | Cluster of differentiation |
| CDC | Complement-dependent cell-mediated cytotoxicity |
| cDNA | Complementary DNA |
| CFSE | Carboxyfluoroscein succinimidyl ester |
| CLDN12 | Claudin 12 |
| CLL | Chronic lymphocytic leukaemia |

| | |
|---|---|
| CMV | Cytomegalovirus |
| CR | Complete remission |
| CRBN | Cereblon |
| CRC | CLL Research Consortium |
| $C_T$ | Cycle threshold value |
| CTL | Cytotoxic T-lymphocyte |
| CTLA4 | Cytotoxic T-Lymphocyte Antigen 4 |
| CTNNA1 | α-catenin |
| CXCL | CXC Motif Ligand |
| CXCR | CXC Motif Receptor |
| Cy7 | Cyanine 7 |
| CYFIP | Cytoplasmic FMR1 interacting protein 1 |
| DAG | Diacylglycerol |
| DAPI | 4’,6-diamidino-2-phenylindole |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| DTH | Delayed type hypersensitivity |
| EDTA | Ethylenediamine tetra-acetate |
| ELISA | Enzyme-linked immunosorbant assay |
| EOMES | Eomesodermin |
| ERK | Extracellular signal-regulated kinases |
| F11R | F11 receptor (=JAMA; = JAM1) |
| FAM | 6-carboxyfluoroscein |
| FasL | Fas Ligand |
| FCR | Fludarabine, cyclophosphamide, rituximab |
| FCS | Foetal calf serum |
| FITC | Fluorescein isothiocyanate |
| FLIPL | FLICE-like inhibitory protein long form |
| FMNL1 | Formin-like protein 1 |
| FMO | Fluorescence minus one |
| Foxp3 | Forkhead box P3 |
| FR | Fludarabine, rituximab |
| FSC | Forward scatter |
| Gads | GRb2-like adapter downstream of Shc |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |

| | |
|---|---|
| gDNA | Genomic DNA |
| GCLLSG | German CLL study group |
| GEF | Guanine exchange factor |
| GTP | Guanosine triphosphate |
| GVHD | Graft-versus-host disease |
| GVL | Graft-versus-leukaemia/lymphoma |
| HCDR3 | Third complementarity-determining region of the heavy chain |
| HIPAA | Health Insurance Probability and Accountability Act |
| HIV | Human immunodeficiency virus |
| HLA | Human leucocyte antigen |
| HVEM | Herpes virus entry mediator |
| ICAM | Intercellular adhesion molecule |
| ICOS | Inducible T-cell costimulator |
| IFNγ | Interferon-γ |
| IGVH | Immunoglobulin heavy chain variable region |
| IGVL | Immunoglobulin light chain variable region |
| IL | Interleukin |
| IL7R | Interleukin 7 receptor |
| IMiD | Immunomodulatory drug |
| $IP_3$ | Inositol trisphosphate |
| IRB | Institutional Review Board |
| IRF4 | Interferon regulatory factor 4 |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| IVT | *In vitro* transcription |
| JAM | Junctional adhesion molecule |
| JNK | Jun N-terminal kinase |
| KLRG1 | Killer cell lectin-like receptor subfamily G member 1 |
| LAG3 | Lymphocyte-activation gene 3 |
| LAMP1 | Lysosomal-associated membrane protein 1 |
| LAT | Linker of activated T-cells |
| Lck | Lymphocyte-specific protein tyrosine kinase |
| LC-MS | Liquid chromatography mass spectrometry |
| LFA1 | Leucocyte function antigen 1 |
| LKB1 | Liver kidney B1 |
| LN | Lymph node |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| MAP | Mitogen-associated protein |
| MAPK | Mitogen-associated protein kinase |
| MBL | Monoclonal B-cell lymphocytosis |
| MDM2 | Mouse double minute 2 homolog |
| MDS | Myelodysplastic syndrome |
| MGB | Minor Groove Binder |
| MHC | Major histocompatibility complex |
| MM | Multiple Myeloma |
| mTOR | Mammalian target of rapamycin |
| NFAT | Nuclear factor of activated T-cells |
| NFκB | Nuclear factor κ light chain enhancer of activated B cells |
| NFQ | Non-fluorescent quencher |
| NLCs | Nurse-like cells |
| NK | Natural killer |
| NOS | Nitric oxide synthase |
| ORR | Overall response rate |
| OS | Overall survival |
| PB | Peripheral blood |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD1 | Programmed death-1 |
| PDL1 | Programmed death ligand-1 |
| PDL2 | Programmed death ligand-2 |
| PE | Phycoerythrin |
| PerCP | Peridinin-chlorophyll-protein |
| PFS | Progression free survival |
| PI3K | Phosphoinositide 3-kinase |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| PKCθ | Protein kinase C θ |
| PLCγ | Phospholipase γ |
| PMA | Phorbol myristate acetate |
| PP2A | Protein phosphatase 2A (also known as protein phosphatase 2) |
| PP2B | Protein phosphatase 2B (also known as protein phosphatase 3) |

| | |
|---|---|
| PPP2R3C | Protein phosphatase 2, regulatory subunit B, gamma |
| PVDF | Polyvinylidene fluoride |
| QRT-PCR | Quantitative Real Time Polymerase Chain Reaction |
| Rap1 | Ras-related protein 1 |
| RasGRP | RAS guanyl nucleotide-releasing protein |
| REC | Research Ethics Committee |
| RHAMM | Receptor for hyaluronan-mediated motility |
| RHOA | Ras homolog gene family, member A |
| RHOC | Ras homolog gene family, member C |
| RIC | Reduced intensity conditioning |
| RIN | RNA integrity number |
| RNA | Ribose nucleic acid |
| RND1 | Rho family GTPase 1 |
| ROR1 | Receptor tyrosine kinase–like orphan receptor 1 |
| RORγt | Retinoic acid orphan receptor γt |
| SEB | Staphylococcal enterotoxin B |
| SEREX | Serological Identification of Recombinantly Expressed Clones |
| siRNA | Short interfering RNA |
| SLL | Small lymphocytic lymphoma |
| SLP76 | Src homology 2 domain-containing leukocyte protein of 76 kDa |
| SMAC | Supramolecular activation cluster |
| SOCS1 | Suppressor of cytokine signalling 1 |
| SSC | Side scatter |
| STAT | Signal transducers and activators of transcription |
| TAA | Tumour-associated antigen |
| TBET | T box expressed in T-cells |
| TBS | Tris buffered saline |
| TBS-T | Tris buffered saline with Tween |
| Tc1 | T cytotoxic 1 cell |
| Tc2 | T cytotoxic 2 cell |
| Tc17 | T cytotoxic 17 cell |
| TCL1 | T cell leukaemia/lymphoma 1 |
| $T_{CM}$ | Central memory T-cell |
| TCR | T-cell receptor |
| $T_{EM}$ | Effector memory T-cell |

| | |
|---|---|
| $T_{EMRA}$ | Effector memory T-cell with expression of CD45RA |
| Tfh | Follicular helper T-cell |
| TGFβ | Transforming growth factor-β |
| Th1 | T helper 1 cell |
| Th2 | T helper 2 cell |
| Th9 | T helper 9 cell |
| Th17 | T helper 17 cell |
| Th22 | T helper 22 cell |
| TIM3 | T cell immunoglobulin mucin-3 |
| TJP2 | Tight junction protein 2 (= ZO2) |
| TNFα | Tumour necrosis factor α |
| TPM2 | Tropomyosin 2 (beta) |
| TRAIL | Tumour necrosis factor-related apoptosis-inducing ligand |
| $T_{reg}$ | Regulatory T-cell |
| TSQ | Triple stage quadrupole |
| VEGF | Vascular endothelial growth factor |
| WASF1 | Wiskott-Aldrich syndrome protein family member 1 |
| WASp | Wiskott-Aldrich syndrome protein |
| WAVE2 | WASp family-verprolin homologous protein 2 |
| XIAP | X-linked inhibitor of apoptosis protein |
| ZAP70 | Zeta-chain-associated protein kinase 70 |
| ZO2 | Zona occludens 2 |

# Chapter 1: Introduction

## 1.1 Background

It is now understood that the immune system plays a crucial role in the development of cancer. In addition to the importance of the innate inflammatory response in promoting this disease, the ability of malignant cells to evade immune destruction has been identified as an "emerging hallmark" of cancer in a recent prominent review (Hanahan and Weinberg 2011). The concept of cancer immune-surveillance appears to be particularly important in cancers of the immune system, due to the potential for high levels of interaction between the tumour cells and the non-malignant immune cells. This is highlighted by the clinical observation that reconstitution of an intact immune response, in the form of allogeneic haematopoietic stem cell transplantation, can be curative for a range of haematological malignancies. The fact that this graft-versus-lymphoma/leukaemia effect appears to be primarily mediated by T cells underlies the concept of repairing T-cell responses as a potentially potent therapeutic modality (Kolb 2008).

B-cell chronic lymphocytic leukaemia (CLL) is a disease caused by a clonal expansion of small, mature B lymphocytes. The immune-surveillance hypothesis would suggest that in order to produce a clinically detectable tumour mass, the malignant B cells must have evolved strategies of evading or suppressing the immune system, especially the anti-cancer effects of T cells (Dunn *et al.* 2002). Surprisingly, it has been known for several decades that T cells are actually expanded in CLL, with a range of functional defects (Catovsky *et al.* 1974; Chiorazzi *et al.* 1979). It still remains unclear whether this expansion reflects a defective immune response, or whether these T cells have been co-opted by the tumour cells to form a vital part of the cancer microenvironment. An understanding of T-cell dysfunction should allow for the development of strategies that repair T-cell anti-tumour responses, resulting in an "autograft versus leukaemia effect". Furthermore, T cells provide "help" to B cells as part of normal immune responses, by stimulating the B cells to proliferate, inducing B-cell antibody class switching and promoting plasma cell differentiation. It could be postulated that in CLL, the T cells have been skewed to provide "help" for the malignant B cells. Therefore in addition to a direct anti-tumour effect, successful immune reconstitution should also have an indirect

effect, by reducing the availability of T-cell derived pro-CLL factors with resultant "starvation" of the CLL cells. Even in the absence of any anti-tumour effects, immune reconstitution could be beneficial to patients by enabling them to fight infections more effectively, and counteract the immune suppression induced by both the disease and by current therapies. Strategies employing this approach should result in therapies that are both more tolerable to vulnerable patients, and show enhanced efficacy in the more challenging poor risk subgroups. This thesis will aim to characterise the nature of the T-cell defect that is present in CLL, and investigate the potential for repairing this defect.

## 1.2 Normal T-cell function

### *1.2.1 T-cell Development and Activation*

The life of a T cell begins as a lymphocyte progenitor derived from pluri-potent haematopoietic stem cells in the bone marrow. These progenitors subsequently migrate to the thymus, and it is there that all the important stages in their development occur: hence the name thymus-dependent (T) lymphocyte. After migrating to the thymus these lymphoid progenitors undergo expansion to generate a large population of immature T cells that do not express either the CD4 or CD8 co-receptor: *double-negative* CD4-CD8- T cells. Approximately 98% of these immature T-cells will subsequently die before they leave the thymus. The fate of each individual CD4-CD8- T cell depends largely on the outcome of the rearrangement of its T-cell receptor (TCR) genes, with cells that express a TCR with either too-high or too-low an affinity for self-peptide:self-MHC complexes undergoing cell death by apoptosis, in processes known as positive or negative selection. Positive selection essentially selects for T cells capable of interacting with self-MHC, with only those T cells that bind self-peptide:self MHC complexes with adequate affinity receiving the necessary signals to survive. T-cell co-receptor expression is also determined during this process with cells with TCRs that can recognise MHC class II molecules becoming $CD4^+$ cells, while cells with TCRs that can recognise MHC class I molecules maturing into $CD8^+$ cells. In contrast, negative selection removes T cells that bind too strongly to self-peptide:self-MHC, and is a critical step in preventing autoimmunity. While the thymus is the source of large numbers of new T cells in the foetus and juvenile, the development of new T cells in the thymus diminishes in adults. Here peripheral expansion plays a greater role, with T-cell numbers being maintained by both the presence long-lived T cells, together with the division of mature T cells outside the central lymphoid organs (Murphy *et al.* 2008). Once T cells have completed their development and have left the thymus, they continuously re-circulate between the blood, lymphatics and lymphoid tissues. This continuous circulation allows them maximal chance of encountering their specific antigen. Mature T cells that have not encountered their specific antigen are known as naive T cells and usually express CD45RA. Naive T cells can live for many years, infrequently divide or undergo apoptosis, and have a low rate of RNA/protein synthesis. Morphologically, they are small with condensed chromatin and scanty cytoplasm. To participate in an adaptive immune response, a naive T cell must meet its specific antigen, presented to it as a peptide:MHC complex on the surface of an antigen

presenting cell (APC) - typically a macrophage, B-cell or dendritic cell, in a process known as priming. Engagement of the T-cell receptor (TCR) by the peptide:MHC complex induces the T cells to proliferate and differentiate into effector cells that clear the antigen. APCs deliver three kinds of signal for clonal expansion and differentiation of naive T cells (Murphy *et al.* 2008):

- Signal 1: TCR engagement + CD4/CD8 co-receptor: ACTIVATION
- Signal 2: Co-stimulatory molecules: SURVIVAL
- Signal 3: Cytokines: DIFFERENTIATION

Once a T cell has been activated, it re-enters the cell cycle and divides rapidly to produce the large numbers of daughter cells that become effector T cells, in a process known as clonal expansion. The cytokine interleukin-2 (IL2) is critical for this proliferation and differentiation, and is produced by the activated T cell itself, along with the alpha chain of the IL2 receptor, CD25. TCR engagement by the peptide:MHC complex causes clustering of the TCR complex and CD4/CD8 co-receptors. The kinases Fyn and Lck phosphorylate tyrosine residues on the CD3ε and ζITAMs, allowing zeta-chain-associated protein kinase 70 (ZAP70) to bind. Lck activates ZAP70, which in turn phosphorylates the linker for the activation of T cells (LAT) and src homology 2 domain-containing leukocyte protein of 76 kDa (SLP76). SLP76 binds and activates phospholipase C-γ (PLCγ) which in turn cleaves phosphatidylinositol biphosphate ($PIP_2$) to yield diacylglyceral (DAG) and inositol triphosphate ($IP_3$). DAG activates both protein kinase C-θ (PKCθ) and Ras guanyl nucleotide-releasing protein (RasGRP), which activate the transcription factors nuclear factor-κB (NFκB) and activated protein-1 (AP1) (via a MAP kinase cascade) respectively. $IP_3$ increases the intracellular calcium concentration, activating a phosphatase, calcineurin, which in turn activates nuclear factor of activated T cells (NFAT)(Smith-Garvin *et al.* 2009).

It is these transcription factors, NFAT, AP1, and NFκB, which regulate cell differentiation and proliferation, including upregulation of IL2 production by binding to the promoter region of the IL2 gene. Co-stimulation through CD28 contributes to the production of IL2 in at least two ways. First, signals from CD28 increase the production of AP1 and NFκB, which increases the initiation of transcription of IL2 mRNA approximately threefold. The second effect of CD28 signalling is thought to be the stabilisation of IL2 mRNA, which increases the production of IL2 protein 20-30-fold. These two effects together allow co-stimulation by CD28 to increase IL2 production as

much as a 100-fold compared with TCR engagement alone (Appleman *et al.* 2000). When a T cell recognizes specific antigen in the absence of co-stimulation through its CD28 molecule, relatively small amounts of IL2 are produced and the T cell does not proliferate. This mechanism plays an important role in avoiding auto-immune responses. Although many autoreactive T cells undergo clonal deletion during their development in the thymus, some potentially autoreactive T cells will remain, as not all self-proteins are presented by the APCs in the thymus. To protect against the induction of T-cell responses against tissue-specific proteins, there is a relative absence of co-stimulatory activity on tissue cells. Therefore, T-cells recognizing self-peptides on tissue cells are not activated, instead they are thought to become anergic, becoming refractory to activation by the specific antigen even when it is subsequently presented by an APC expressing co-stimulatory molecules. These anergic T cells may play an important role in preventing responses by naive, non-anergic T cells to foreign antigens that mimic self-peptide:self-MHC complexes. Anergic T cells could recognize and bind to such complexes on APCs without responding, and thus could compete with the potentially autoreactive naive T cells of the same specificity. Another explanation is that the anergic cells are in fact a form of regulatory T cell, as there are some similarities in the phenotype (Schwartz 2003).

### *1.2.2 T-cell Activation and the Immunological Synapse*

A key part of T-cell activation after recognition of a peptide:MHC complex is formation of an immunological synapse. This involves polymerisation of F-actin and polarisation of the cytoskeleton, resulting in the redistribution of TCRs, signalling, and adhesion molecules into a region beneath the T-cell:APC contact site (Huppa and Davis 2003). These molecules are segregated into supramolecular activation clusters (SMACs), the central (cSMAC), peripheral (pSMAC) and distal (dSMAC) respectively (**Figure 1.1**). The cSMAC contains a concentration of TCR:CD3:peptide:MHC complexes, costimulatory molecules such CD28, and signalling molecules such as PKCθ. The pSMAC is enriched with adhesion molecules such as lymphocyte function-associated antigen 1 (LFA1), and the dSMAC contains large glycoproteins such as CD45 (Grakoui *et al.* 1999). In the case of $CD8^+$ cytotoxic T-lymphocytes (CTLs) there is also recruitment of cytotoxic granules into the central area of the synapse, allowing targeted release and cell death (Huppa and Davis 2003).

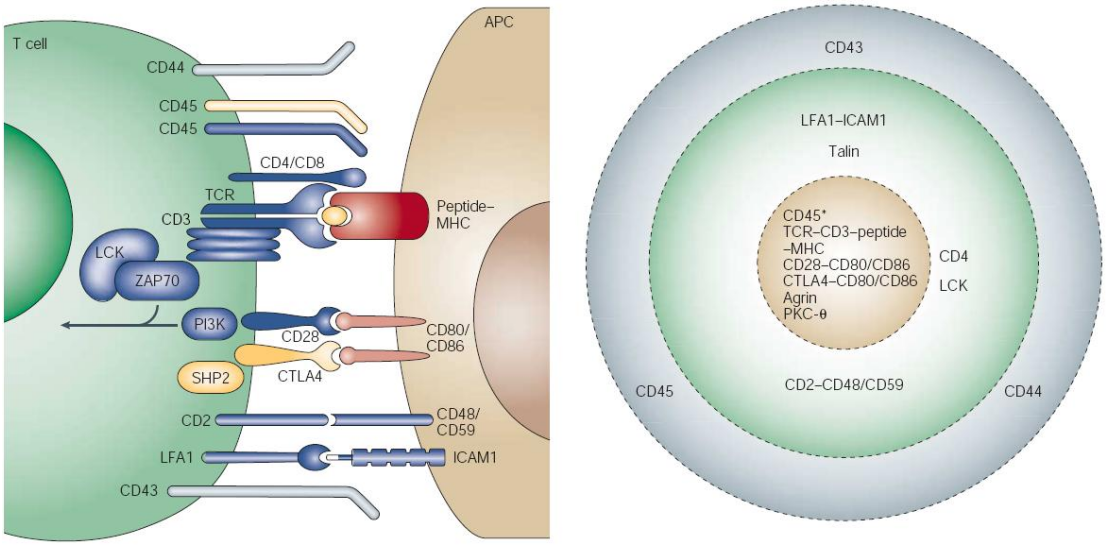



**Figure 1.1 Overview of a mature immunological synapse (Huppa and Davis 2003)**

The rearrangement of the actin cytoskeleton is an active process which is regulated by TCR-signalling. An important bridge between upstream Lck/ZAP70 signalling and downstream events such as calcium flux, Ras activation, and phosphatidylinositol cleavage is LAT. LAT is phosphorylated by ZAP70, allowing it to bind the adaptor protein GRb2-like adapter downstream of Shc (Gads) and recruit the scaffold protein SLP76 to the activation complex. After phosphorylation by ZAP70, SLP-76 binds the adaptor protein, Nck, and the guanine nucleotide exchange factor (GEF), Vav. Nck recruits the actin nucleation factor Wiskott-Aldrich Syndrome Protein (WASp) to the

TCR site, and Vav activates the Rho GTPases, cdc42 and Rac1 (Rossman *et al.* 2005). These RhoGTPases regulate actin filament polymerisation through three different classes of actin nucleation factors: the actin related proteins 2/3 (Arp2/3) complex (activated by WASp and WASp family-verprolin homologous protein 2 (WAVE2)), the formins, and the spire proteins (Reicher and Barda-Saad 2010).

The polarisation of the actin cytoskeleton and formation of the immunological synapse plays a pivotal role in antigen recognition, signal transduction, T-cell proliferation, migration, adhesion, and tissue invasion. The synapse appears to act as a type of adaptive controller that can both amplify and attenuate T-cell receptor signalling (Lee *et al.* 2003). Endocytosis of the TCR is an important component of this control, as the TCR has been shown to be downregulated after formation of the cSMAC (Lee *et al.* 2003). The actin cytoskeleton also regulates integrins such as LFA1 which undergoes clustering and conformational changes upon T-cell activation. These changes rapidly enhance its affinity and avidity for its ligand, intracellular adhesion molecule (ICAM), enabling stable T-cell:APC interactions and efficient activation. The actin cytoskeleton is highly involved in the signalling pathways that lead to integrin activation. In particular WAVE2 is thought to play a major role, both by activating the small GTPase, Ras-proximity-1 (Rap1) and through the recruitment of the integrin scaffolding protein talin, which binds integrins in a Rap1 dependent manner (Nolz *et al.* 2007; Smith-Garvin *et al.* 2009).

### *1.2.3 T-cell Differentiation*

Our understanding of T-cell differentiation has evolved considerably over the last decade. The traditional paradigm developed during the 1980s hypothesised that T-cells could be divided into functional subsets according their co-receptor expression ($CD4^+$ versus $CD8^+$) and cytokine production. $CD8^+$ T-cells were all thought to differentiate into cytotoxic T cells that were able to kill their target cells and were important in the defence against intracellular pathogens such as viruses. In contrast, $CD4^+$ T-cells could differentiate into two principle subsets that provided "help" for other immune cells. The basis for the concept of classifying these two subtypes had come from observations of the heterogeneous course of some infectious diseases such as leprosy. The healing (tuberculoid) form of leprosy was found to be associated with strong delayed-type hypersensitivity (DTH) reactions and relatively low levels of antibody, whereas uncontrolled (lepromatous) leprosy was associated with high antibody titres and weak DTH (Turk and Bryceson 1971). It was subsequently demonstrated that mouse $CD4^+$ T-cells could be classified into two distinct populations on the basis of their patterns of cytokine production: Th1 cells that produced IFNγ, IL2, and TGFβ and mediated DTH reactions, and Th2 cells that produced IL4, IL5, IL6, and IL13 and mediated humoral immunity (Mosmann *et al.* 1986; Fiorentino *et al.* 1989). This paradigm not only provided a simple explanation for the dichotomy of the clinical observations, but also provided other key concepts for an understanding of T-cell differentiation. Each T-cell subset was shown to produce cytokines that serve as their own autocrine growth factor and promote differentiation of naive T cells to that subset (Lichtman *et al.* 1987). Furthermore, the two subsets were shown to produce cytokines and modulate transcriptional activity to cross-regulate the other's development and activity.

However, while certain aspects of this paradigm have held true, it has become apparent that the situation is considerably more complex. A naive T cell, whether $CD4^+$ or $CD8^+$, has an increasing variety of functional fates open to it upon activation. These are determined by integration of a number of different signals: the strength of signalling from the TCR, the presence or absence of co-stimulation, the cytokine milieu in which the T cell finds itself, and small molecule modifiers. Both naive $CD4^+$ and $CD8^+$ T-cells can develop into effector or memory cells, with memory cells being further subdivided into effector-memory or central-memory. Within the effector subsets there are a range of phenotypes defined both by the principle cytokine produced, and whether they have

an immuno-regulatory function. The cytokine milieu in which a naive T-cell recognises antigen is particularly critical, with the resultant effector phenotype in both $CD4^+$ and $CD8^+$ T cells being determined certain cytokines: for example IL12 and IFNγ for Th1/Tc1, IL-4 for Th2/Tc2, and IL-6 and TGFβ for Th17/Tc17 (Mosmann *et al.* 1986; Maggi *et al.* 1994; Mosmann *et al.* 1997; Jager and Kuchroo 2010). In many of the $CD4^+$ subtypes, the cytokines have been shown to upregulate specific transcription factors, which act as "master controllers" of the various differentiation pathways (Table 1) (Zheng and Flavell 1997; Szabo *et al.* 2000; Hori *et al.* 2003; Ivanov *et al.* 2006; Nurieva *et al.* 2009) (**Table 1.1**). There is emerging evidence that TBET, GATA3, RORγt, may play a similar role in regulating differentiation of $CD8^+$ T cells resulting in Tc1, Tc2, and Tc17 cells respectively (Apte *et al.* ; Szabo *et al.* 2000; Curtis *et al.* 2009; Hamada *et al.* 2009).

| T-cell subtype | Differentiation factor | Transcription factor |
|---|---|---|
| Th1/Tc1 | IL12 | TBET |
| Th2/Tc2 | IL4 | GATA3 |
| Th9 | IL4, TGF | PU.1 |
| Th17/Tc17 | IL6, TGFβ, then IL23 | RORγt |
| Tfh | IL21 | Bcl6 |
| Treg | IL2 | FoxP3 |

**Table 1.1: Cytokines and transcription factors regulating T-cell differentiation**

The mechanisms underlying the interaction between cytokines, signal-transducing activator of transcription (STAT) signalling and transcription factors are best understood in Th1/Th2 differentiation in $CD4^+$ cells. This is summarised in **Figure 1.2** (Amsen *et al.* 2009). These two distinct pathways of helper T-cell differentiation are driven by the cytokines IL12 and IL4 and the transcription factors TBET and GATA3 respectively. Engagement of the TCR by its specific peptide:MHC complex initially induces low grade expression of the IFNγ (*IFNG*) and TBET (*TBX21*) genes. When IL12 is present, STAT4 signalling increases the expression of IFNγ which bind to the IFNγ receptor in an auto/paracrine manner. This activates STAT1 which strongly promotes expression of the *TBX21* gene. The resultant upregulation of the transcription factor TBET has a number of important effects including a further increase in IFNγ production, increased expression of the IL12 receptor β-chain, and also prevention of Th2 differentiation by directly inhibiting GATA3 (Szabo *et al.* 2000; Mullen *et al.* 2001; Hwang *et al.* 2005).

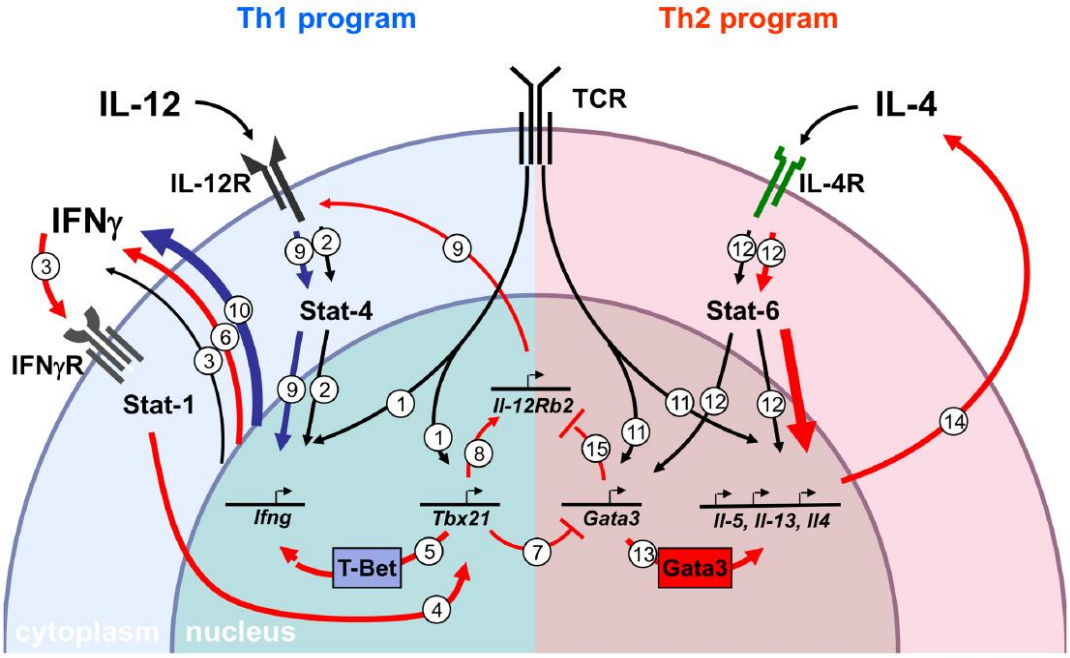



**Figure 1.2 Regulation of Th1/Th2 differentiation (Amsen *et al.* 2009)**

In contrast, Th2 development is directed by IL4. Initial activation of TCR-signalling in the presence of IL4 causes activation of STAT6, which promotes expression of the *GATA3* and *IL4* genes. The transcription factor GATA3 reorganises chromatin structure in the Th2 locus, which encompasses the *IL4*, *IL5* and *IL13* genes, increasing their translational competence. Increased production of IL4 further enhances Th2 differentiation in a positive feedback loop. GATA3 prevents Th1 differentiation by inhibiting expression of the IL12 receptor β-chain and the *STAT4* gene (Zhang *et al.* 1997; Zheng and Flavell 1997; Yamashita *et al.* 2004; Kim *et al.* 2007).

Similarly, there also appears to be a reciprocal relationship between Th17 cells and Tregs. Th17 cells, so called because of their production of the effector cytokine IL17, were the third class of $CD4^+$ helper T cells to be described (Langrish *et al.* 2005). They are thought to be particularly important in the defence against certain extracellular bacteria such as *Citrobacter*, *Klebsiella pneumoniae*, and *Borrelia burgdorferi*, and fungi such as *Candida albicans* (Bettelli *et al.* 2008). IL17 also appears to play an important role in tissue inflammation, and thus Th17 cells heavily implicated in autoimmune diseases, particularly those affecting the central nervous system (Langrish *et al.* 2005; Ivanov *et al.* 2006). It was initially suggested that IL23 was the differentiation factor for Th17 cells, but subsequent work has shown that their

differentiation is driven by TGFβ and IL6 (Bettelli *et al.* 2006). IL6 signalling leads to the phosphorylation of STAT3, and ultimately to expression of RORγt, which is believed to regulate many components essential for the differentiation of Th17 cells including IL17A, IL17F, and the IL23 receptor (Ivanov *et al.* 2006; Yang *et al.* 2007).

While Th17 cells have been found to promote tissue inflammation and autoimmunity, activation and expansion of self-reactive T-cells is suppressed by "naturally occurring" $CD4^+CD25^+$ regulatory T cells ($T_{regs}$) (Sakaguchi *et al.* 1995; Sakaguchi 2000). It was subsequently demonstrated that the transcription factor, FoxP3, plays a key role in regulating the development of these cells (Hori *et al.* 2003). In the same way that there is a reciprocal relationship between Th1 and Th2 cells, there appears to be a similar relationship between Th17 cells and $T_{regs}$. TGFβ alone is anti-inflammatory and is essential for the development of $T_{regs}$, but the presence of pro-inflammatory IL6 has been shown to inhibit the induction of $FoxP3^+$ $T_{regs}$, and simultaneously promote Th17 differentiation (Bettelli *et al.* 2006). It was subsequently demonstrated at the molecular level that RORγt and FoxP3 can bind to each other and inhibit each other's function (Zhou *et al.* 2008). Furthermore, IL2, a growth factor for $T_{regs}$, inhibits Th17 differentiation through activation of STAT5, whereas IL21, which promotes Th17 differentiation, inhibits the expansion of $T_{regs}$ (Laurence *et al.* 2007). Retinoic acid has also been shown to promote $T_{reg}$ differentiation while inhibiting Th17 cells (Mucida *et al.* 2007).

The situation is further complicated by the emergence of another helper T-cell subset, Th9 cells. In contrast to Th17 cells and $T_{regs}$, these are induced by the combination TGFβ and IL4 and primarily produce IL9 and IL10 (Jager and Kuchroo 2010). The transcription factor PU.1, previously shown to be essential for many aspects of lymphoid and myeloid cell development, has recently emerged as being required for the development of this subset (Chang *et al.* 2010). They appear to be effector rather than regulatory, and have been implicated in the development of allergic inflammation, particularly in the lungs (Carotta *et al.* 2010).

### *1.2.4 Transcriptional Control of Effector/Memory Development*

In addition to their role as controllers of T-cell differentiation, some of these transcription factors also regulate the development of effector and memory T cells. In contrast to the effector T cells described above, memory T cells are long-lived and persist after an infection has been cleared. They are so-called because of their ability to undergo rapid expansion to generate large numbers of effector T cells upon re-exposure to their specific antigen, thus providing the immune system with *memory* against past infections. Memory T cells can be divided into two main subtypes on the basis of distinct expression of chemokine receptors (CCRs) or homing molecules: $CD44^+CD62L^+CCR7^+$ central memory T cells ($T_{CM}$ cells) and $CD44^+CD62L^-CCR7^-$ effector memory T cells ($T_{EM}$ cells) (Sallusto *et al.* 1999). $T_{CM}$ cells mediate what has been called "reactive memory", with their constitutive expression of CCR7 and CD62L allowing them to home to secondary lymphoid organs. They have little or no effector function, but in comparison to naive T cells show greater sensitivity to antigenic stimulation, are less dependent on co-stimulation, and up-regulate CD40L to a greater extent. However, upon recognition of their cognate antigen, they have a high proliferative potential and can rapidly differentiate into large numbers of effector cells (Sallusto *et al.* 2004).

In contrast, $T_{EM}$ cells mediate what has been called "protective memory", by migrating to inflamed peripheral tissues and displaying immediate effector function. $CD8^+$ $T_{EM}$ cells carry large amounts of perforin, and both $CD4^+$ and $CD8^+$ $T_{EM}$ cells can produce IFNγ, IL4 and IL5 within hours after following antigenic stimulation. Within the CD8+ $T_{EM}$ subtype, there is a subset that expresses CD45RA and contains large amounts of perforin, termed $T_{EMRA}$ cells (Sallusto *et al.* 2004). This subset also shows reduced expression of co-stimulatory molecules such as CD27 and CD28, and has been found to be enriched in CMV infection (Hamann *et al.* 1997; Champagne *et al.* 2001; Appay *et al.* 2002). Interestingly, while all memory T cells show a relatively low threshold for activation, the potential for expansion progressively decreases from $T_{CM}$ cells to $T_{EM}$ cells, with $T_{EMRA}$ cells having a particularly low capacity for proliferation and expansion (Geginat *et al.* 2003). This correlates with a decrease in telomere length and an increased tendency to undergo apoptosis. These constraints can be overcome by co-stimulation, which has been shown to both induce telomerase activity, and upregulate anti-apoptotic Bcl-XL (Boise *et al.* 1995; Son *et al.* 2000).

Under normal circumstances the relative proportions of $T_{CM}$ and $T_{EM}$ cells in blood are associated with their co-receptor expression: $T_{CM}$ cells are predominant in the $CD4^+$ compartment and $T_{EM}$ cells in the $CD8^+$ compartment. Within the tissues, $T_{CM}$ and $T_{EM}$ cells show characteristic patterns of distributions with $T_{CM}$ cells enriched in lymph nodes and tonsils, whereas lung, liver and gut contain greater proportions of $T_{EM}$ cells (Campbell *et al.* 2001). Significantly, $T_{CM}$ cells appear to be heterogeneous in their ability to differentiate into a Th1 or Th2 phenotype and this has been shown to correlate with expression of CXCR3 and CCR4 respectively. A proportion of circulating $T_{CM}$ cells express CXCR5, the receptor for CXCL13, which is a chemokine produced in B cell follicles, and hence have been called follicular helper T cells (Tfh-cells). They are produce IL2, IL10, and IL21 upon activation, and when present in tonsils express CD40L, ICOS, CD84 and CD200 providing spontaneous help to B cells (Breitfeld *et al.* 2000; Schaerli *et al.* 2000; Chtanova *et al.* 2004; Vogelzang *et al.* 2008). IL21 has been found to be the principle differentiation factor, and has been demonstrated to upregulate the transcription factor BCL6, which regulates Tfh-cell development (Nurieva *et al.* 2008; Nurieva *et al.* 2009; Stewart 2009).

During an immune response, naive T cells undergo a pronounced clonal expansion during which large numbers of antigen-specific T cells are generated. This initial phase of expansion and acquisition of effector functions is followed by a contraction phase where the majority of the reactive T cells undergo apoptosis, leaving behind a small, but relative stable population of memory T cells. As described above, when these cells re-encounter their specific antigen they can efficiently proliferate and undergo rapid transition into effector cells. Traditionally memory T cells were considered to arise during the contraction phase, and were felt to directly develop from effector cells. However, observations that memory cells display many features that are more characteristic of naive T-cells, along with the discovery of the IL7 receptor α-chain (IL7R; CD127) expression as a hallmark for memory cells and their precursors, conflicts with this model. Instead, memory T cells are thought to diverge early during an immune response, and arise in parallel with short-lived effector cells (Kallies 2008).

In line with this "fixed lineage model" of differentiation, there is evidence that the cytokine milieu also determines distinct transcriptional profiles of effector and memory T-cells, and that these are imprinted early during the immune response. A number of transcription factors have been implicated in this process, including TBET,

Eomesodermin (EOMES), BCL6 and BLIMP1. Like TBET, EOMES is also a member of the T-box family, and together these transcription factors have been demonstrated to regulate $CD8^+$ T-cell effector and memory differentiation. They have been shown to enhance expression of IL2Rβ, a component of the IL15 receptor, and therefore enhance the IL15 signalling required for the survival of memory T cells (Intlekofer *et al.* 2005). Interestingly, while deficiency of just one of these two transcription factors does not appear to significantly affect $CD8^+$ T-cell effector/memory differentiation, absence of both transcription factors leads to aberrant differentiation into IL17 producing $CD8^+$ T-cells (Intlekofer *et al.* 2008). Evidence is emerging that differentiation of $CD8^+$ T-cells occurs in two principle phases, and the mammalian target of rapamycin (mTOR) plays a key role in regulating their fate. The mTOR kinase senses environmental cues, nutrient availability, internal energy stores, and growth factor signalling to regulate cell growth, proliferation and differentiation (Powell and Delgoffe 2010). Inhibition of mTOR by rapamycin has been previously demonstrated to induce a state of anergy in $CD4^+$ T cells, and has also been shown to induce $CD4^+Foxp3^+$ $T_{regs}$ (Powell *et al.* 1999; Haxhinasto *et al.* 2008). In naive $CD8^+$ T-cells, mTOR plays an essential role in regulating TBET versus EOMES expression. During the early stages of infection, high amounts of antigen, inflammation and the presence of cytokines such as IL12 resulted in increased and sustained expression of TBET and mTOR activity, promoting the differentiation of $T_{EM}$ cells. As levels of antigen fall in the setting of decreased inflammation, decreased mTOR activity switches the transcriptional program from TBET to EOMES promoting the differentiation of $T_{CM}$ cells (**Figure 1.3**) (Shrikant *et al.* 2010).

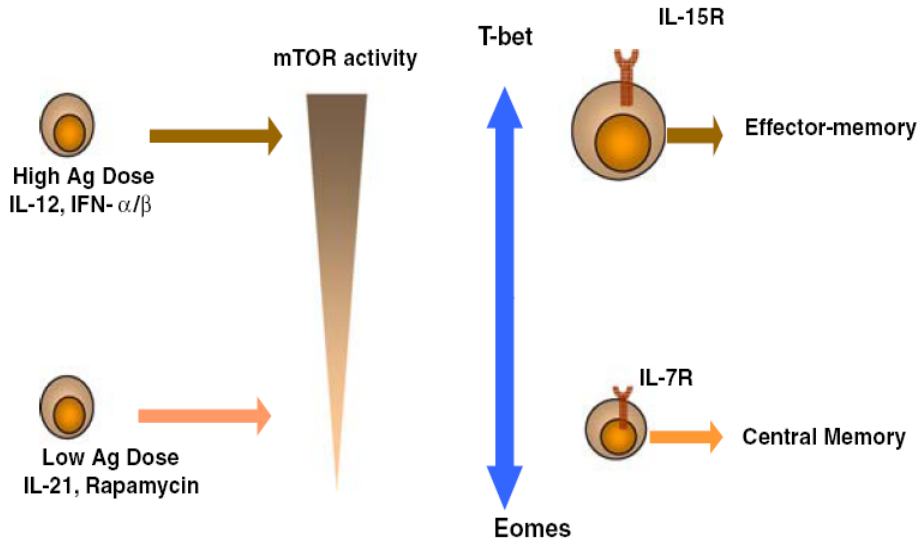



**Figure 1.3 Regulation of $CD8^+$ T cell effector and memory differentiation**

This highlights the importance of the concept of strength of TCR signalling, with strong signals promoting the development of an effector phenotype, while weaker signals promote memory cell differentiation. Of note, $CD8^+$ T-cells that have been treated with rapamycin, with enhanced memory-like attributes, demonstrate significantly enhanced activity to control tumour growth, which correlates with observations that $T_{CM}$ cells are required for durable and effective anti-tumour immune responses (Shrikant *et al.* 2010).

The expression of TBET and EOMES also correlates with the surface expression of IL7R. Both naive and memory T cells express high levels of IL7R, but this molecule is downregulated by most T cells in the early stages after antigenic stimulation. A subpopulation of these cells will regain expression of IL7R during the course of the immune response, and this population has a high capacity to become memory T cells. TBET has been shown to directly repress expression of the *IL7R* gene, thus promoting effector cell differentiation at the expense of memory cell formation (Joshi *et al.* 2007; Kallies 2008). In contrast, ectopic T-bet expression by OT1 $CD8^+$ T cells down-regulated CD127 and SOCS1, and upregulated CD122 and IL15Rα, switching the cellular survival cytokine profile from IL7 to IL15 (Yeo and Fearon 2011).

The transcription factors BCL6 and BLIMP1 have also been implicated the regulation of memory CD8+ T-cell development. BLIMP1 was originally shown to regulate the terminal differentiation of B cells into antibody secreting plasma cells (Turner *et al.* 1994). Bcl6 was initially cloned from patients with non-Hodgkin lymphoma, and it was subsequently shown to be crucial for B cell memory development in germinal centres (Ye *et al.* 1993; Fukuda *et al.* 1997). Overexpression of BLIMP1 has been shown to suppress T-cell proliferation and may be important in terminating the immune response by promoting activation induced cell death (AICD). BLIMP1 is expressed most strongly in the later stages of T-cell differentiation and its expression may block memory differentiation (Kallies 2008). In contrast, BCL6 has been shown to promote the development of $T_{CM}$ cells, and may work in part by antagonising the AICD promoting activity of BLIMP1 (Ichii *et al.* 2002; Kallies 2008).

### *1.2.5 Chronic Antigenic Stimulation and T-cell Exhaustion*

Significantly, there are significant differences in $CD8^+$ memory T-cell differentiation in acute and chronic viral infections. As described above, in acute infections antigen stimulation results in the production of activated $CD8^+$ T-cells which proliferate extensively and acquire effector functions such as cytokine production and cytotoxicity, along with chemokine production and new migratory properties. The majority of the effector $CD8^+$ T cells then die during the contraction phase, with about 5 – 10% remaining as memory $CD8^+$ T cells (Angelosanto and Wherry 2010). In contrast during chronic viral infections, the effector T cells are unable to differentiate into functional memory cells, and become dysfunctional as the infection progresses. Exhausted $CD8^+$ T cells progressively lose the ability to produce IL2, have reduced capacity to proliferate, show reduced cytotoxicity, and have a greater propensity to undergo apoptosis (**Figure 1.4**). Eventually, fully exhausted $CD8^+$ T cells can even lose the ability to produce cytokines including IFNγ (Zajac *et al.* 1998; Fuller and Zajac 2003; Wherry *et al.* 2003). A further characteristic is the of exhausted $CD8^+$ T cells is the upregulation of inhibitory receptors including PD1, LAG3, CD160, and 2B4 (Barber *et al.* 2006; Blackburn *et al.* 2009).

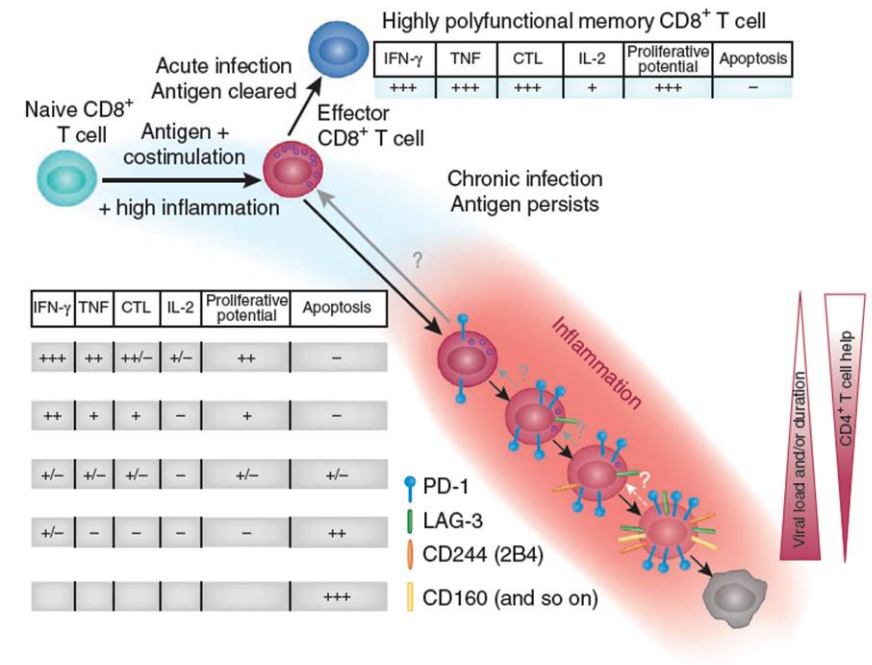



**Figure 1.4 T-cell exhaustion (Wherry 2011)**

Some functions of these exhausted T cells can be regained by blocking inhibitory receptor pathways, such as PD1 and/or LAG3, but the greater the number of inhibitory receptors expressed by a cell, the more severe the exhaustion and the less chance of regaining function (Blackburn *et al.* 2008; Blackburn *et al.* 2009). Antigen independent

memory T-cell maintenance can also become compromised during chronic viral infection, with exhausted $CD8^+$ T-cells responding poorly to IL7 and IL15. Instead virus-specific $CD8^+$ T cells generated during chronic infection require antigen to survive, and the pattern of division for maintenance is distinct from memory $CD8^+$ T cells generated after acute infection (Shin *et al.* 2007).

Programmed cell death 1 (PD1) is a known T-cell inhibitory receptor, with expression of its ligand, PDL1, on non-haematopoietic cells, playing an important role in the maintenance of peripheral tolerance and preventing autoimmunity (Sharpe *et al.* 2007). Its expression on T-cells is inducible, being upregulated within 24-72 hours of stimulation. This axis has been exploited by a variety of infectious organisms, such as HIV, to attenuate antimicrobial immunity and facilitate chronic infection, and has also been implicated in cancer (Weber 2010). Engagement of PD1 by its ligands during TCR signalling can block T-cell proliferation, cytokine production and cytolytic function, and impair T-cell survival. The extent of PD1 inhibition depends on the strength of the TCR signal, with more inhibition occurring at lower levels of TCR stimulation. CD28 co-stimulation or IL2 can override PD1 mediated inhibition (Francisco *et al.* 2010). PD1 engagement has a number of effects on TCR signalling, including inhibition of PI3K/Akt activity, and prevention of the induction of BclxL and transcription factors such as GATA-3, TBET and Eomes (Nurieva *et al.* 2006). The expression of the two ligands, PDL1 and PDL2 is regulated by inflammatory milieu, with cytokines such as the IFNγ and TNFα upregulating their expression on T cells, B cells, endothelial cells, and epithelial cells (Keir *et al.* 2008).

The transcription factor BLIMP1 has emerged as playing a key role in $CD8^+$ T-cell exhaustion during chronic viral infection (Shin *et al.* 2009). It has been shown to be dramatically upregulated in virus specific $CD8^+$ T-cells as these cells became exhausted. It has also been shown to both upregulate inhibitory receptors such as PD1, and also repress key molecules involved in normal memory $CD8^+$ T-cell differentiation, including IL7R and CD62L. NFAT has also been implicated, with a defect in its nuclear translocation having been demonstrated to be responsible for defective cytokine production by exhausted $CD8^+$ T-cells (Agnellini *et al.* 2007). Under normal circumstances these transcription factors may act to regulate cytotoxicity in exhausted T cells whilst avoiding excess damage from release of pro-inflammatory cytokines (Angelosanto and Wherry 2010).

## 1.3 Chronic lymphocytic leukaemia

### *1.3.1 Chronic lymphocytic leukaemia*

B-cell chronic lymphocytic leukaemia (CLL) is a disease caused by a clonal expansion of small, mature $IgM^+/IgD^+$ B lymphocytes. It is the commonest leukaemia in the western world, and is a disease of the elderly with a median age of 72 years at diagnosis (SEER Cancer Factsheet)(2012). CLL has characteristic immunophenotype with co-expression of the T-cell antigen CD5 and B-cell antigens CD19, CD20, and CD23. The levels of surface immunoglobulin, CD20 and CD79b are generally decreased compared to those found on normal B cells (**Table 1.2**). The diagnosis of CLL is dependent on the presence of $\geq 5 \times 10^9$/L monoclonal lymphocytes with this phenotype in the peripheral blood persisting for more than 3 months (**Figure 1.5**). The disease can also present with lymphadenopathy, splenomegaly, hyper-metabolic symptoms such as weight loss, fevers and night sweats, and evidence of bone marrow failure. The term small lymphocytic leukaemia (SLL) is used where there is lymphadenopathy and/or splenomegaly due to an infiltration of clonal B cells with the same phenotype as CLL, but with a circulating lymphocytosis $< 5 \times 10^9$/L (Hallek *et al.* 2008; Swerdlow 2008).

**Table 1.2 CLL Scoring System**

| **Marker** | | **(Score)** | | **(Score)** |
|---|---|---|---|---|
| SmIg | Weak | (1) | Moderate/Strong | (0) |
| CD5 | Positive | (1) | Negative | (0) |
| CD23 | Positive | (1) | Negative | (0) |
| FMC7 | Negative | (1) | Positive | (0) |
| CD79b | Weak | (1) | Strong | (0) |
| **Total Score for CLL: 3 – 5; scores for non-CLL cases: 0 – 2** | | | | |

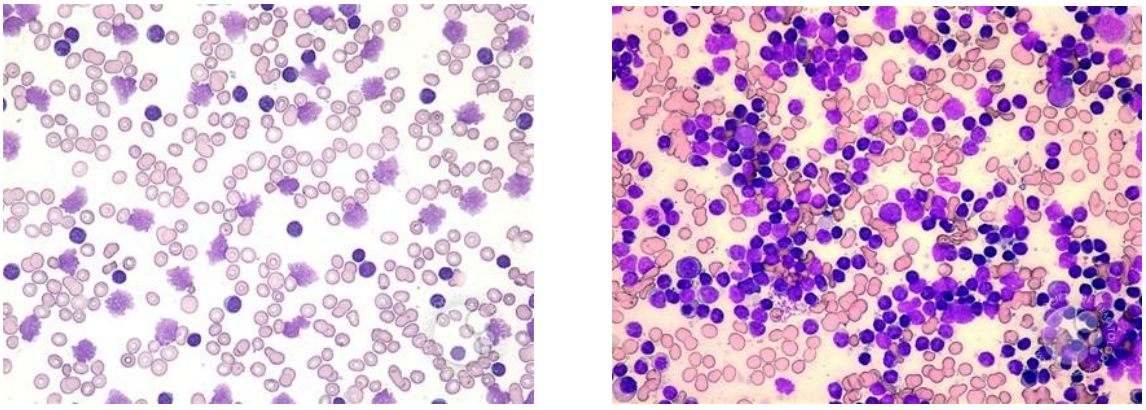


**Figure 1.5 Peripheral blood smear and bone marrow aspirate in CLL**

### *1.3.2 Prognostic Stratification*

CLL is a very heterogeneous disease. The clinical course can range from indolent, where patients may never require treatment, to aggressive, where patients have rapid progression of disease in the peripheral blood, bone marrow, and lymphoid organs. This heterogeneity is maintained after treatment, with a wide variation between patients in terms of depth of response to treatment and response duration.

Several prognostic markers have been identified that identify patients with poorer risk disease. Classically, assessment of risk was based on clinical and basic laboratory parameters. The Rai and Binet staging systems are the most widely used, in both patient care and clinical trials (Rai *et al.* 1975; Binet *et al.* 1981). The original Rai classification had five prognostic groups before it was modified to three groups, such that both systems now have three major subgroups with discreet clinical outcomes (**Table 1.3**).

**Table 1.3 Rai and Binet staging systems in CLL**

| **Rai modified staging** | | | | **Binet clinical staging** | | |
|---|---|---|---|---|---|---|
| **Risk** | **Stage** | **Clinical features** | **Median survival** | **Stage** | **Clinical features** | **Median survival** |
| Low | 0 | Lymphocytosis alone | >12.5 yrs | A | No anaemia or thrombocytopenia <3 lymphoid regions enlarged | 12 yrs |
| Intermediate | I | Lymphocytosis & lymphadenopathy | 8.4 yrs | B | No anaemia or thrombocytopenia 3 or more lymphoid regions enlarged | 5 yrs |
| | II | Lymphocytosis & spleno/hepatomegaly | 5.9 yrs | | | |
| High | III | Lymphocytosis & anaemia (< 11.0 g/dL) | 1.6 yrs | C | Anaemia (< 10.0 g/dL) and/or thrombocytopenia (< 100 x $10^9$/L) | 2 yrs |
| | IV | Lymphocytosis & thrombocytopenia (< 100 x $10^9$/L) | 1.6 yrs | | | |

Over the last 10 – 15 years several other prognostic markers have emerged, based on the biological characteristics of the disease. The most extensively used and studied of these include *IGVH* gene mutation status, ZAP70 and CD38 expression, and cytogenetics (Chiorazzi 2012). Cytogenetic abnormalities as defined by fluorescence *in situ* hybridisation are the most frequently used and widely available biological prognostic markers for the clinical care of CLL patients. A landmark paper published in 2000 documented the most frequent genetic alterations present in CLL and correlated them with outcome (Dohner *et al.* 2000). On the basis of a regression analysis of 325 patients, a hierarchical model of chromosomal aberrations was defined that was predictive of both median survival and time to first treatment. Five categories were defined: 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as the sole abnormality; the median survival times for patients in these groups were 32, 79, 114, 111, and 133 months, respectively. Patients with deletion of either 17p or 11q were also noted to have the most clinically advanced disease as defined by the Rai and Binet criteria, as well as more extensive lymphadenopathy and splenomegaly. Ongoing work has identified the critical gene(s) within the affected regions in these chromosomes (**Table 1.4**).

**Table 1.4 Critical genes implicated within chromosomal abnormalities in CLL**

| Chromosomal abnormality | Critical genes within the affected region | Reference |
|---|---|---|
| Deletion 13q14 | *DLEU2*: codes for miR15a and miR16-1 | (Calin et al. 2002) |
| Trisomy 12 | *NOTCH1* | (Balatti *et al.* 2012) |
| Deletion 11q22-23 | *RDX; ATM* | (Stilgenbauer *et al.* 2002) |
| Deletion 17p | *TP53* | (Zenz *et al.* 2010) |

*IGVH* gene mutation status is a second important prognostic marker in CLL. Under normal circumstances, naive B cells are continuously circulating through the blood and lymphatic system. Upon encountering their cognate antigen, the activated B cells migrate to lymph node primary follicles, where along with their associated T cells, they proliferate and form a germinal centre. As part of this "germinal centre reaction", these B cells undergo somatic hypermutation of the V regions of their immunoglobulin genes, which in turn allows for the selection of mutated B cells with increased affinity for the antigen (affinity maturation). In just over half (~55%) of CLL cases, the malignant B cells show evidence of having been through this process, as sequencing of their *IGVH* genes shows < 98% sequence homology to germline ("mutated"). In the other 45% of

cases the malignant B cells have features of naive B cells, in that their *IGVH* genes show > 98% sequence homology to germline ("unmutated"), suggesting that the cell of origin had not passed through the germinal centre reaction. These two categories were associated with radical differences in prognosis, with patients with mutated *IGVH* genes in the tumour cells having a significantly better outcome than patients with unmutated *IGVH* genes (Hamblin *et al.* 1999). It has been postulated that this may be due to differences in the affinity of the BCR for autoantigens in these two situations. Cases with mutated *IGVH* genes may have CLL cells with BCRs that have higher affinity for a more restricted range of antigens than unmutated cases, which may lead to either anergy where antigens are present, or death by neglect where antigens are absent (Chiorazzi 2012). High expression of CD38 and ZAP70 are both correlated with unmutated *IVGH* genes, and are predictive of a poorer prognosis (Damle *et al.* 1999; Crespo *et al.* 2003). Both $CD38^+$ and $ZAP70^+$ cases show evidence of a higher degree of cellular activation of the clone, with evidence of more recent proliferation and greater responsive to surface immunoglobulin cross-linking in $CD38^+$ cases, and prolongation of BCR-signalling in $ZAP70^+$ cases (Zupo *et al.* 1996; Chen *et al.* 2002; Damle *et al.* 2002; Lanham *et al.* 2003; Pepper *et al.* 2006; Calissano *et al.* 2009). Overall, a picture is emerging where patients with aggressive disease have "weakly anergic" tumour cells that have higher capacity to respond to BCR-signalling and proliferate, which corresponds to high expression of CD38 and ZAP70 and unmutated *IVGH* genes. In contrast, patients with more indolent disease have "strongly anergic" tumour cells, which are relatively quiescent, with low expression of CD38 and ZAP70, and mutated *IGVH* genes.

### *1.3.3 Standard Treatments*

In standard practice, newly diagnosed patients with early stage disease (Rai 0, Binet A) should be monitored without therapy unless they have evidence of disease progression. Treatment guidelines state that therapy should be reserved for those with advanced, symptomatic or progressive disease, as a meta-analysis of more than 2000 patients with early disease showed no survival benefit with early treatment with alkylating agents (1999; Hallek *et al.* 2008). Patients with Binet stage B or C, or Rai intermediate or high risk disease usually benefit from treatment. The following criteria are useful in determining the need for treatment initiation:

- Evidence of progressive bone marrow failure
- Massive, progressive, or symptomatic splenomegaly
- Massive, progressive, or symptomatic lymphadenopathy
- Progressive lymphocytosis with an increase of more than 50% over a two-month period or lymphocyte doubling time of less than six months.
- Autoimmune anaemia/thrombocytopenia unresponsive to standard therapy
- Constitutional symptoms of weight loss, night sweats, fevers or fatigue

Combination therapy with fludarabine, cyclophosphamide and rituximab (FCR) has emerged as the current standard of care for CLL. The German CLL Study Group (GCLLSG)-led CLL8 study showed that patients treated with this combination achieved significant improvements in progression free survival (PFS) and overall survival (OS) compared to patients treated with fludarabine and cyclophosphamide (FC) alone, with overall response rates (ORR) of 95% (Hallek *et al.* 2010). Despite these encouraging results, FCR is not curative, with complete remission (CR) rates of only 44%, and a median progression free survival (PFS) of 52 months. Furthermore, this regimen is also not suitable for all patients. FCR has a significant side effect profile, and appears too toxic for the elderly and those with co-morbidities. The GCLLSG also conducted a randomized phase III trial comparing upfront therapy with *single agent* fludarabine against chlorambucil in patients over 65 years of age. A total of 193 patients with a median age of 70 years were enrolled. Despite higher ORR and CR rates, this did not translate into improved PFS or OS (Eichhorst *et al.* 2009). This is highly relevant given that two-thirds of patients are over 65 years of age at the time of diagnosis. Even in the younger age-group, high risk patients such as those with 17p deletion, or who have treatment-refractory or rapidly relapsed disease, represent a major challenge. Therefore there is an urgent need for novel agents in CLL.

### *1.3.4 Molecular Targeted Therapies*

Given the success of the tyrosine kinase inhibitors in chronic myeloid leukaemia (CML) there has been increasing interest in developing more targeted therapies for CLL. Many of the most recent therapeutic developments directed at the CLL B cells have focused on signalling through the B-cell receptor (BCR). BCR signalling is known to be crucial for B-cell proliferation and survival, and a number of major prognostic markers that are clinically useful in CLL are associated with aberrations in BCR signalling, as described above. The BCR consists of a surface immunoglobulin (Ig) molecule non-covalently associated with an Igα/Igβ (CD79a/CD79b) heterodimer. In normal B cells, engagement of the BCR by antigen induces phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic domains of Igα and Igβ. This phosphorylation is primarily mediated by the Src family kinase Lyn, and results in the recruitment and activation of the tyrosine kinase Syk. The activated Syk forms a membrane associated complex with other tyrosine kinases such as Lyn and the Tec kinase, Bruton's tyrosine kinase (BTK), and adapter molecules such as B-cell linker protein (BLNK). This complex mediates the activation of downstream signalling pathways, such as phosphatidyl 3-kinase (PI3K), and phospholipase Cγ2 (PLCγ2). PI3K generates the second messenger phosphatidylinositol-3,4,5-triphosphate ($PIP_3$), which recruits molecules such as the kinase Akt. PLCγ2 activation leads to the release of intracellular calcium and subsequent activation of protein kinase C (PKC). These events lead in turn to activation of mitogen activated protein kinases (MAPKs) including extracellular signal regulated kinase (ERK), c-JUN $NH_2$-terminal kinase (JNK) and p38 MAPK. Activation of PKC also increases expression of the transcription factor nuclear factor-κB (NFκB), while the rise in the intracellular calcium concentration causes activation nuclear factor of activated T cells (NFAT). It is likely that the expression profile of these transcription factors is fundamental in determining B-cell fate (Niiro and Clark 2002).

One of the most striking aspects of CLL biology is that the immunoglobulin variable heavy (*IgVH*) and light chain genes (*IgVL*) have a very limited repertoire, with highly similar gene rearrangements being demonstrated in a number of patients. This suggests that chronic antigenic drive plays a critical role in the pathogenesis of this disease, conferring a selective growth advantage to the malignant cells (Rosen *et al.* 2010). In light of the of the importance of BCR signalling in promoting CLL, there has been a

large amount of interest in targeting key molecules in this signalling pathway. A variety of molecules that inhibit the activity of downstream kinases have been developed, including inhibitors of Lyn, Syk, BTK and PI3K (**Figure 1.6)**. Two of these, the BTK-inhibitor ibrutinib (PCI-32765) and the PI3K-inhibitor idelalisib (CAL-101; GS-1101) look particularly exciting and are in advanced stage clinical trials with/without other agents.

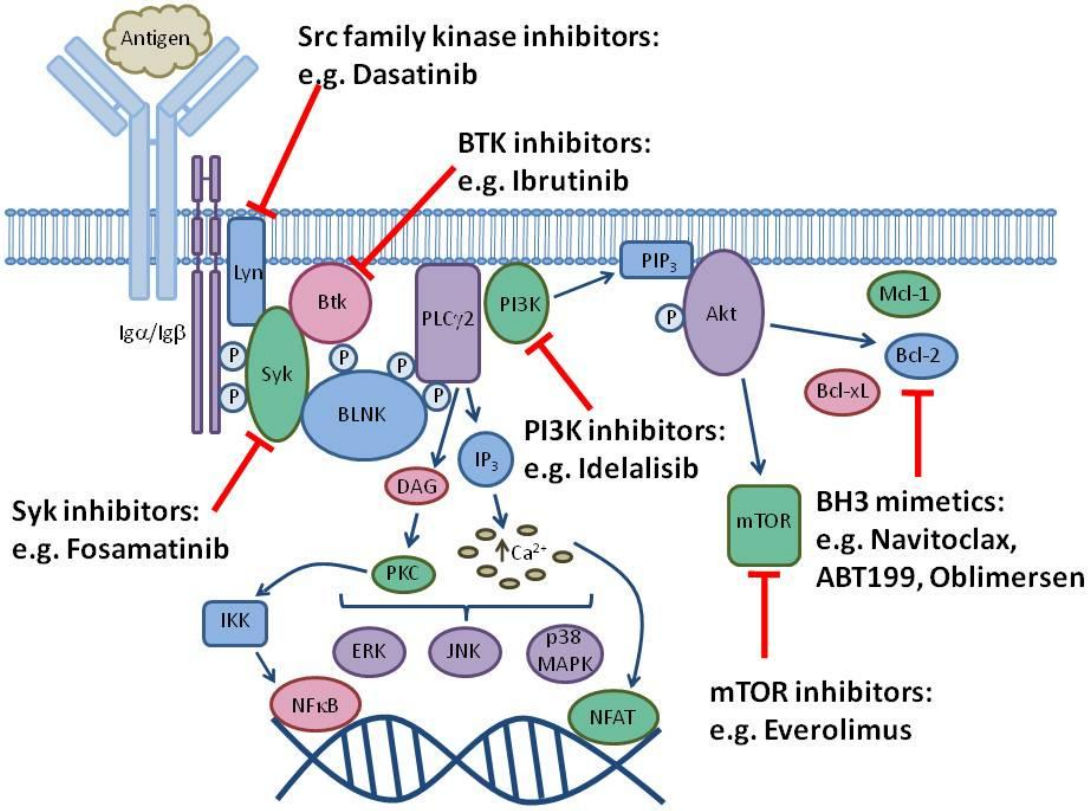



**Figure 1.6 BCR-signalling pathway and targets for molecular inhibitors**

Pre-clinical studies of the BTK inhibitor, ibrutinib, showed that it is directly cytotoxic to primary CLL cells, dependent on caspase activation. This agent also appears to have effects on the microenvironment, by blocking the protective effect of factors such as CD40 ligand (CD40L), TNFα, IL6, and B cell activating factor (BAFF) (Herman *et al.* 2010). A recently published clinical phase Ib/II study of two-dose cohorts (420mg/840mg) of ibrutinib in patients with relapsed or refractory CLL/SLL showed ORRs of 71% in both cohorts, with PFS of 75% and OS of 83% at 26 months. Intriguingly, one of the defining characteristics of ibrutinib treatment was rapid lymph node shrinkage associated with an *increase* in the circulating lymphocytosis, consistent with mobilisation of the CLL cells from the tissues to the blood. This lymphocytosis complicated the analysis of response by standard criteria: if an additional category of

response with lymphocytosis was included, a further 20% and 15% of patients in the two dose cohorts could be considered to respond. Ibrutinib was well tolerated with minimal haematological toxicity (Byrd *et al.* 2013).

Inhibitors of PI3K are also under investigation as potential treatments for CLL. The PI3K pathway has been shown to play a pivotal role in CLL-cell growth, with sustained activation being critical for their survival (Cuni *et al.* 2004). Constitutive activation of this pathway prevented the downregulation of Bcl-xL (B-cell lymphoma-xL) and the caspase inhibitor proteins FLICE-inhibitory protein ligand (FLIPL) and X-linked inhibitor of apoptosis protein (XIAP). Idelalisib is a small-molecule inhibitor of the p110δ isoform of PI3K, which is generally restricted to cells of hematopoietic origin. It has been demonstrated to promote caspase dependent apoptosis in primary CLL cells independently of common prognostic markers. Idelalisib has also been shown to overcome the anti-apoptotic effects of CD40L, TNF-α, and fibronectin, and to also inhibit the protective effects of stromal cells (Herman *et al.* 2010). A phase I study of the use of single agent idelalisib in patients with relapsed or refractory CLL has shown it to be well tolerated, with partial remissions being observed in 33% of patients. Interestingly, the drug-mediated mobilisation of lymphocytes from the lymph nodes to the peripheral blood was also observed with this agent (Furman *et al.* 2010).

A further area that has been the subject of recent investigation is the use of agents that inhibit the anti-apoptotic Bcl2 family members, Bcl2, BclxL, and Mcl1. These proteins prevent apoptosis by sequestering the pro-apoptotic proteins Bax and Bak. They are commonly over-expressed in CLL B cells, with evidence that this is due to sustained BCR-signalling and prolonged activation of the PI3K/Akt and MEK/ERK pathways (Gottardi *et al.* 1996; Longo *et al.* 2008). Several inhibitors of the Bcl2 family have been developed, which inhibit one or more of these molecules. The first-generation Bcl2 inhibitor, navitoclax, achieved partial remissions (PRs) in 35% of patients with relapsed/refractory CLL (Roberts *et al.* 2009). However, this agent also inhibited BclxL expressed in platelets, resulting in dose-limiting thrombocytopenia. ABT-199 is a second generation Bcl2 inhibitor that has greater affinity for Bcl2 but 500-fold less affinity for Bcl-xL. Preliminary data from a phase I study of this agent in patients with relapsed/refractory CLL have shown impressive efficacy, with 85% ORR including 88% responses in patients with deletion of 17p and 78% responses in fludarabine-refractory patients (Roberts *et al.* 2013).

## 1.4 The Immune System in CLL

Immune dysfunction is a key feature of CLL, highlighted by the hypogammaglobulinaemia, increased susceptibility to infections, and increased incidence of autoimmune cytopenias that are commonly seen in this disease. As a consequence, attempts to understand nature of the immune defect in CLL have been active areas of investigation. The immune-surveillance hypothesis would suggest that in order to produce a clinically detectable tumour mass, the malignant B cells must have evolved strategies of evading or suppressing the immune system, especially the anti-cancer effects of T lymphocytes (Dunn *et al.* 2002). An understanding of these mechanisms and the subsequent immune cell defects, should allow the development of new therapeutic approaches aimed at restoring anti-tumour immune responses.

### *1.4.1 T-cell Dysfunction in CLL: Abnormalities in T-cell Numbers*

It has been known for some time that the T-cell compartment is highly abnormal in CLL, with one of the earliest studies reporting an increase in absolute numbers of T cells in the PB (Catovsky *et al.* 1974). This expansion is primarily accounted for by an increase in $CD8^+$ T cells, resulting in a fall in the CD4:CD8 ratio,(Lauria *et al.* 1980; Herrmann *et al.* 1982; Mills *et al.* 1982; Platsoucas *et al.* 1982) in contrast with the situation in the LNs and bone marrow, where increased numbers of $CD4^+$ T cells have been reported (Pizzolo *et al.* 1983). The mechanism behind these changes remains unclear, although it has been suggested that the relative reduction in numbers of PB $CD4^+$ T cells is due to their increased susceptibility to FasL-mediated apoptosis (Tinhofer *et al.* 1998). A number of studies have linked changes in the numbers of circulating T cell numbers to prognosis. Early work demonstrated that there was greater expansion of $CD8^+$ T cells, reflected in falling CD4:CD8 ratios, with advancing Rai stage (Herrmann *et al.* 1982). A more recent study has shown that inverted CD4:CD8 ratios predict shorter time to first treatment and reduced progression-free survival (Nunes *et al.* 2012). Furthermore, other investigators have shown that patients with "pre-malignant" monoclonal B cell lymphocytosis (MBL) appear to lack any significant expansion in T cell numbers, in contrast to patients with CLL (Te Raa *et al.* 2012). However, there may be difficulties trying to prognosticate based on just the PB microenvironment, as another study found that increased numbers of circulating $CD8^+$ correlate with longer median time of survival (Gonzalez-Rodriguez *et al.* 2010).

### *1.4.2 T-cell Dysfunction in CLL: Abnormalities in Cytokine Secretion Profiles*

Despite their increased numbers, these T cells show profound defects in function and proliferative capacity, and abnormal cytokine secretion profiles (Chiorazzi *et al.* 1979; Lauria *et al.* 1983; Prieto *et al.* 1993). Early studies suggested that CLL was a Th2/Tc2 mediated disease, with increased numbers of $CD4^+$ and $CD8^+$ T cells producing interleukin-4 (IL4).(Mu *et al.* 1997; Mainou-Fowler *et al.* 2001) Further work showed that these IL4-producing T cells also upregulate CD30 by an IL4 and OX40L-dependent mechanism (de Totero *et al.* 1999; Cerutti *et al.* 2001). Interaction of CD30 with CD30L expressed on the CLL cells results in increased TNFα production and CLL-cell proliferation. In contrast, ligation of CD30L on the surface of the non-malignant B cells impairs isotype class switching, and increases their sensitivity to FasL-mediated cell death, potentially contributing to the hypogammaglobulinaemia observed in CLL. IL4 also appears to be able to protect the CLL cells from undergoing apoptosis, by increasing/maintaining bcl2 protein levels (Dancescu *et al.* 1992; Panayiotidis *et al.* 1993). There is evidence to suggest that interleukin-6 (IL6) production by CLL cells drives this skew towards IL4 producing T cells. Healthy T cells stimulated in the presence of tumour supernatant containing high levels of IL6 have been shown to increase their production of IL4, findings that could be replicated using recombinant IL6, or reversed by removal or blockade of this cytokine (Buggins *et al.* 2008). Furthermore, IL6 has been demonstrated to be increased in the serum of patients with CLL, with higher levels predicting poorer survival (Kurzrock *et al.* 1993; Fayad *et al.* 2001).

This shift towards a type 2 T-cell response was an attractive hypothesis given that these T cells could be expected to both enhance humoral immunity and "help" the malignant B cells, while suppressing Th1/Tc1 mediated cellular anti-tumour immune responses. However, a number of other reports have described increased production of IFNγ and TNFα by T cells from CLL patients, showing correlation with disease stage (Zaki *et al.* 2000; Bojarska-Junak *et al.* 2002; Gallego *et al.* 2003; Podhorecka *et al.* 2004; Kiaii *et al.* 2005) Furthermore, IFNγ and TNFα have been shown to protect CLL cells from apoptosis and induce their proliferation *in vitro* (Digel *et al.* 1989; Buschle *et al.* 1993). It is probable that the situation *in vivo* is more complicated than the traditional Th1/Th2 paradigm, with interplay between a variety of cell types producing a mix of cytokines, chemokines, and cell surface molecules that protect the CLL cells from apoptosis and regulate their proliferation (**Table 1.5**).

**Table 1.5 Cytokines implicated in the pathogenesis of CLL**

| Cytokine | Action | Reference |
|---|---|---|
| IFN-γ | Protects CLL cells from apoptosis | (Buschle *et al.* 1993) |
| TNF-α | Increases CLL cell proliferation | (Digel *et al.* 1989) |
| TGF-β | Inhibits CLL cell proliferation | (Lotz *et al.* 1994) |
| IL-2 | Increases CLL cell proliferation | (Yoshizaki *et al.* 1982) |
| IL-4 | Protects CLL cells from apoptosis | (Dancescu *et al.* 1992; Panayiotidis *et al.* 1993) |
| IL-5 | Induces CLL cell apoptosis | (Mainou-Fowler *et al.* 1994) |
| IL-6 | Protects CLL cells from apoptosis; inhibits CLL cell proliferation | (Aderka *et al.* 1993; Reittie *et al.* 1996) |
| IL-7 | Increases CLL cell proliferation; protects CLL cells from apoptosis | (Yoshioka *et al.* 1992; Long *et al.* 1995) |
| IL-10 | Inhibits CLL cell proliferation; induces CLL cell apoptosis | (Fluckiger *et al.* 1994; Tangye *et al.* 1998) |
| IL-21 | Induces CLL cell apoptosis | (di Carlo *et al.* 2007) |

### *1.4.3 T-cell Dysfunction in CLL: Chronic Activation and the Role of CMV?*

Another feature of CLL T cells is that they show evidence of chronic activation, with upregulation of activation markers such as CD69, HLA-DR and CD57, and down-regulation of CD28 and CD62L (Velardi *et al.* 1985; Rossi *et al.* 1996; Van den Hove *et al.* 1998; Van den Hove *et al.* 1998). Several reports document expansions of clonal and oligoclonal T cells in CLL, in both $CD4^+$ and $CD8^+$ populations (Wen *et al.* 1990; Farace *et al.* 1994; Rezvany *et al.* 1999; Goolsby *et al.* 2000). There is also evidence that these oligoclonal expansions are primarily restricted to populations with an activated $CD57^+$ phenotype, suggesting a role for chronic antigen stimulation in their development (Serrano *et al.* 1997). However, it remains unclear as to whether this is directly related to CLL, or whether other factors such as cytomegalovirus (CMV) are involved. CMV is known to have a profound influence on distribution of lymphocyte subsets in healthy individuals, with CMV sero-positivity driving the T-cell repertoire towards greater clonality in the elderly (Khan *et al.* 2002; Chidrawar *et al.* 2009). Subsequent reports have demonstrated an expansion of CMV-specific $CD4^+$ and $CD8^+$ T cells in patients with CLL, with an effector phenotype (Mackus *et al.* 2003; Pourgheysari *et al.* 2010; Walton *et al.* 2010). It is therefore possible the earlier reports of clonality and increased expression of activation markers merely reflect an immune response to CMV.

### *1.4.4 T-cell Dysfunction in CLL: cytoskeletal dysfunction and inhibitory ligands*

An important finding in the immuno-biology of CLL was the work of my group demonstrating profound changes in the global gene expression profiles of T cells from CLL patients when compared with healthy age-matched controls (Gorgun *et al.* 2005). Despite not being part of the malignant clone, alterations were found in the expression of genes involved in cell differentiation and cytoskeletal formation in patient $CD4^+$ T cells, and in cytoskeletal formation, vesicle trafficking, and cytotoxicity pathways in patient $CD8^+$ T cells. Similar defects in gene expression could be induced in healthy T cells by co-culturing them with CLL cells. These changes in the expression of cytoskeletal genes translated into a functional defect in filamentous actin (F-actin) polymerization, with T cells from CLL patients exhibiting defective immunological synapse formation with antigen presenting cells (APCs)(Ramsay *et al.* 2008). Again, this functional defect could be induced in healthy T cells by co-culturing them with CLL cells. In both cases the induction of the defects relied on direct contact between the CLL cells and the T cells, as the defects were not seen after co-culture experiments using the transwell system.

Intriguingly, similar defects could be observed in T cells taken from the Eμ-TCL1 mouse model of CLL. In this model, the *TCL1* gene is placed under the control of a B-cell specific $IgV_H$ promoter and $Ig_H$ Eμ enhancer, resulting in mice that develop normally into adulthood, but then subsequently develop lymphadenopathy and hepatosplenomegaly, with increased numbers of circulating $CD19^+CD5^+IgM^+$ lymphocytes (Bichi *et al.* 2002). Despite this model being dependent on a single transgene, T cells from animals that had developed the disease showed alterations in the expression of cytoskeletal genes, and exhibited dysfunctional immunological synapse formation. Furthermore, infusion of CLL cells into young Eμ-TCL1 mice caused defects comparable to those seen in mice that had clinically apparent leukaemia, demonstrating causality of the CLL cells to induce the T cell defects (Gorgun *et al.* 2009). Interestingly, another study has shown that this model also accurately mimics the shift towards the antigen-experienced phenotype observed in the human disease (Hofbauer *et al.* 2011). This work demonstrated both a skewing of T-cell subsets towards antigen-experienced memory T cells, and development of clonal T-cell populations, consistent with a CLL–dependent antigen-driven skewing of the T cells in these mice.

My supervisors subsequently examined the mechanism by which CLL cells induce these defects in the T cell populations. Using functional screening assays based on actin polymerization responses to APCs, the molecules CD200, CD270 (Herpes virus entry mediator; HVEM), CD274 (Programmed death ligand-1; PDL1), and CD276 (B7-H3) were found to induce impaired immunological synapse formation in both allogeneic and autologous T cells (Ramsay *et al.* 2012). Higher expression of these molecules was observed on CLL cells compared to healthy B cells, with increased expression of CD200 and CD274 correlating with poor prognosis. Notably, signalling axes involving these inhibitory ligands were also able to induce impaired F-actin polymerization in other cancers, suggesting that both hematologic and solid cancer cells use common immunosuppressive mechanisms to suppress T-cell mediated anti-tumour immune responses. Of note, there is evidence that some of the receptors for these inhibitory ligands are increased on CLL T cells. PD1 (CD279) is an inhibitory T-cell receptor that has been postulated to be important in the maintenance of peripheral tolerance. Engagement of PD1 by PDL1 leads to the inhibition of T-cell receptor-mediated lymphocyte proliferation and cytokine secretion (Freeman *et al.* 2000). My group has recently reported that PD1 expression is increased on $CD3^+$ T cells from CLL patients and is an indicator of poor prognosis (Ramsay *et al.* 2012). Another group has reported similar findings, showing that PD1 expression is increased on $CD8^+$ T cells, with its expression predicting a more aggressive clinical course (Nunes *et al.* 2012). However, another report has suggested that PD1 expression is decreased on effector T cells, that are expanded in CLL (Tonino *et al.* 2012).

### *1.4.5 T-cell Dysfunction in CLL: Regulatory T cells*

In addition to increased expression of inhibitory receptors in CLL, there is also an increase in $CD4^+CD25^{hi}$ regulatory T-cells ($T_{regs}$) in CLL. $T_{regs}$ have been shown to inhibit proliferation and cytokine release by conventional $CD4^+CD25^-$ T cells, and increased numbers of $T_{regs}$ are associated with inhibition of anti-tumour immunity in solid cancers (Baecher-Allan *et al.* 2004; Curiel *et al.* 2004). The expansion of $T_{regs}$ in CLL has been postulated to be due to two factors. Firstly, there is evidence of increased formation, facilitated by CD27-CD70 interactions in LN proliferation centres (Jak *et al.* 2009). CD200 expression on the CLL cells may also play a role, as anti-CD200R antibodies promote development of $T_{regs}$, and whereas CD200 blockade significantly decreases $T_{reg}$ numbers (Gorczynski *et al.* 2005; Pallasch *et al.* 2009). Secondly, $T_{regs}$ from patients with CLL have increased resistance to drug induced apoptosis, compared

to $T_{regs}$ from healthy donors, as a consequence of higher expression of Bcl-2 (Jak *et al.* 2009). The degree of increase correlates with disease stage, with the greatest increases found in patients with the most clinically advanced disease (Beyer *et al.* 2005; Giannopoulos *et al.* 2008; D'Arena *et al.* 2011; Piper *et al.* 2011). $T_{reg}$ numbers were also noted to be slightly increased in patients MBL, although lower than patients with CLL (D'Arena *et al.* 2011). The degree of increase in $T_{regs}$ has also been found to be of prognostic significance, with higher numbers of $T_{regs}$ predicting significantly shorter time to first treatment in two studies (Weiss *et al.* 2011; D'Arena *et al.* 2012). $T_{regs}$ also express CD39, with increased expression of this molecule showing correlation with disease severity and prognosis in CLL (Pulte *et al.* 2011; Perry *et al.* 2012). Functionally, higher frequencies of $T_{regs}$ have also been shown to correlate with decreased T-cell responses against viral and tumour antigens (Giannopoulos *et al.* 2008). A further feature of $T_{regs}$ is they express another inhibitory receptor, cytotoxic T-lymphocyte associated antigen-4 (CTLA4; CD152). CTLA4 is a homolog of CD28 and acts both directly, by decreasing phosphorylation of signalling proteins proximal to the T-cell receptor, and indirectly, by competing with CD28 for CD80/CD86 binding (van der Merwe *et al.* 1997; Lee *et al.* 1998). T cells from CLL patients have been shown to have significantly increased expression of both surface and cytoplasmic CTLA4 when compared to healthy controls, with expansion of a $CD4^+CD25^+CTLA4^+$ subset (Motta *et al.* 2005). Furthermore, although CTLA4 is normally upregulated upon T-cell activation, CLL T cells also showed a greater degree of upregulation of CTLA4, and prolonged expression (Frydecka *et al.* 2004). Therefore, it is likely that CTLA4 signalling is yet another inhibitory pathway mediating T-cell dysfunction in CLL, in addition to the PD-1:PD-L1, CD160:HVEM and CD200:CD200R axes (**Figure 1.7**).

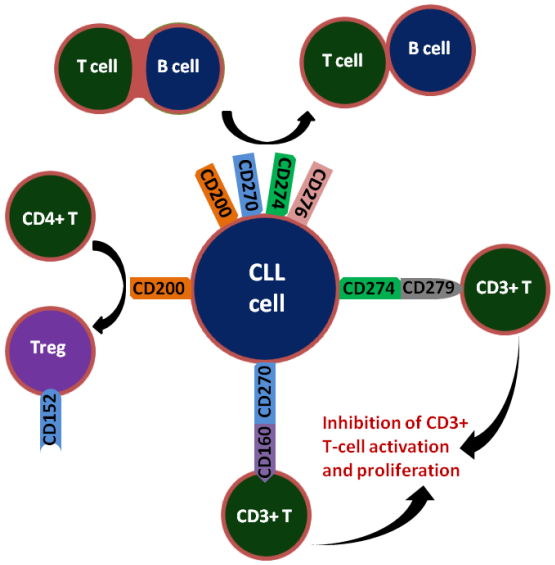



**Figure 1.7 Inhibitory signalling axes in CLL.**

Upregulation of CD200, CD270, CD274 and CD276 induces impaired actin polymerization and immunological synapse formation in CLL T cells. CD200 also promotes the differentiation of $CD4^+$ T cells into $T_{regs}$, which express CD152 (CTLA4). CD274 (PDL1) can interact with its ligand CD279 (PD1) to inhibit T-cell activation and proliferation.

### *1.4.6 T cell Dysfunction in CLL: T cells as Part of the Pro-tumour Niche*

In addition to the role for the $CD4^+CD25^+$ subset in the pathogenesis of CLL, evidence is emerging that implicates $CD4^+$ T cells in the development and maintenance of this disease. As mentioned above, in contrast to the PB where $CD8^+$ T cells are increased, $CD4^+$ T cells are primarily expanded in the LNs and BM of CLL patients (Pizzolo *et al.* 1983). It has been suggested that this is due to production of the chemokine CCL22 by CLL cells, which has been shown to be released into culture supernatants on CLL cell activation. These supernatants were able to induce the migration of activated $CD4^+CD40L^+$ T cells expressing the CCL22 receptor, CCR4: migration which could be blocked by anti-CCL22 antibodies (Ghia *et al.* 2002). A recent paper has highlighted the potential importance of CD4+ T cells in providing a protective niche for engraftment of CLL cells (Bagnara *et al.* 2011). The ability of primary human CLL cells to engraft and proliferate in a non-obese diabetes/severe combined immunodeficiency/$\gamma c^{null}$ mouse model was assessed. Intriguingly, autologous $CD4^+$ T cells from the CLL patients were found to be key mediators of CLL cell growth in these mice, despite representing only a minor component of the transferred lymphocyte population. There was direct correlation between the degree of proliferation of T cells and that of the CLL clone, with selective depletion of $CD4^+$ T cells leading to failure of CLL cell growth. Further

investigation is required to corroborate these findings in CLL patients, but taken together, these observations suggest that the expanded $CD4^+$ T cells seen in BM and LNs are a critical part of the pro-tumour microenvironment.

### *1.4.7 Nurselike cells and chemokines*

Nurselike cells (NLCs) are another key constituent of the immune microenvironment implicated in providing a protective niche for CLL cells. NLCs are a subset of peripheral blood mononuclear cells (PBMCs) that differentiate into large, round, or fibroblast-like adherent cells in the presence of CLL cells (Burger *et al.* 2000). Although they are thought to originate from $CD14^+$ monocytes, their expression of CD68 is significantly higher than normal monocyte-macrophages, making them more comparable to the $CD68^+$ lymphoma-associated macrophages observed in follicular lymphoma (Farinha *et al.* 2005). Further work identified these large $CD14^+CD68^{HI}$ cells in the spleens of CLL patients, suggesting that they might also function to promote leukaemia cell survival *in vivo* (Tsukada *et al.* 2002). An important feature of the biology of CLL cells is that they undergo spontaneous apoptosis when cultured *in vitro*, in marked contrast to their apparent longevity *in vivo* (Collins *et al.* 1989). However, when NLCs are present *in vitro*, the leukemic cells attach to them and are protected from undergoing spontaneous apoptosis. NLCs are thought to promote CLL cell survival due to a combination of secretion of the chemokine CXCL12, and increased expression of B cell-activating factor of the tumour necrosis factor family (BAFF), and a proliferation inducing ligand (APRIL)(Burger *et al.* 2000; Nishio *et al.* 2005). In addition, differentiation of monocytes into NLCs is associated with dysregulation of genes involved in antigen presentation and immunity, impairing their anti-tumour function (Bhattacharya *et al.* 2011). The receptor for CXCL12, CXCR4, is expressed on high levels on the surface of CLL B cells, with its expression correlating with Rai stage (Burger *et al.* 1999; Ghobrial *et al.* 2004). Therefore secretion of CXCL12 by both NLCs and stromal cells induces leukaemia cell trafficking and homing to protective niches populated by these cells. Furthermore, co-culture of CLL cells with NLCs induces the leukemic cells to release two T-cell attracting chemokines, CCL3 and CCL4. The production of these chemokines may explain the observation of increased numbers of T cells in CLL pseudofollicles, where the T cells appear to have been co-opted to "help" B-cells activation and proliferation (Burger *et al.* 2009). Furthermore, increased CCL3 levels correlate with other adverse prognostic markers such as advanced clinical stage and poor risk cytogenetics, and are predictive of shorter time to

first treatment (Sivina *et al.* 2011). In addition, CXCL12 acts as a co-stimulatory factor for CLL $CD4^+$ T cells, resulting in their increased proliferation, cytokine production, and increased expression of activation markers. These activated T cells are then able to enhance the activation and proliferation of the leukemic cells (Borge *et al.* 2012). It is therefore possible that a mix of chemokines including CXCL12, CCL3 and CCL4 results in co-localization of stromal cells, NLCs, and activated $CD4^+$ T cells, that provide "sanctuary sites" for the tumour cells, protecting them from chemo-immunotherapy, resulting in drug resistance and disease persistence (Burger 2010) (**Figure 1.8**).

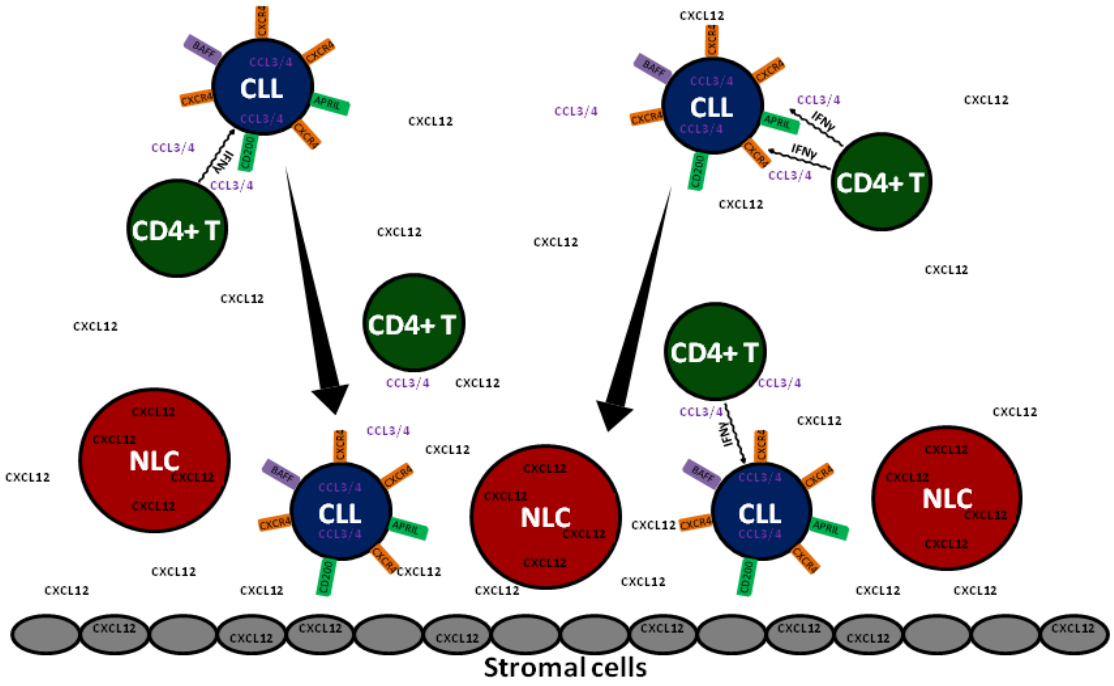



**Figure 1.8 The CLL niche.**

CXCL12 produced by stromal cells and NLCs attracts CXCR4-bearing CLL cells into the protective niche. Interaction of CLL cells with NLCs causes: i) further differentiation of monocytes into NLCs; ii) protection of the CLL cells from apoptosis associated with increased expression of BAFF and APRIL; iii) increased production of CCL3 and CCL4 by CLL cells. CCL3 and CCL4 chemoattract $CD4^+$ T cells which are activated by CXCL12. These activated T cells produce cytokines including IFNγ which regulate CLL cell proliferation and protect them from apoptosis.

### *1.4.8 Natural killer cells*

Abnormalities have also been found in other immune cells in CLL. Initial studies revealed that natural killer cells (NK cells) also have a functional defect in CLL, showing reduced ability to lyse leukaemia cell lines associated with a lack of cytoplasmic granules (Ziegler *et al.* 1981; Kay and Zarling 1984). This activity could be restored by the use of IL2, which also resulted in increased granularity of the large granular lymphocyte subset (Kay and Zarling 1987). The mechanism by which CLL cells downregulate NK-cell function is not known, although there is some evidence that it may involve soluble factors (Burton *et al.* 1989; Katrinakis *et al.* 1996). CLL cells may also inhibit NK cells by direct contact, by their expression of the tolerogenic non-classical MHC class I molecule, HLA-G, or by expression of 4-1BB ligand (Maki *et al.* 2008; Buechele *et al.* 2012). Of note, NK cells from CLL patients show defective actin polymerization and impaired immunological synapse formation, comparable to the cytoskeletal dysfunction seen in CLL T cells (Gaidarova *et al.* 2009). The NK-cell defect appears to be of clinical significance, as higher NK-cell numbers were observed in patients with early stage disease and in those with mutated *IGHV* genes. Furthermore, for patients with a given Rai stage, a higher NK:CLL cell ratio was predictive of a longer time to treatment, implying a protective effect of NK cells (Palmer *et al.* 2008). In support of this, NK cells from patients with MBL have a higher cytolytic capacity than NK cells from CLL patients. (Kimby *et al.* 1989) Other studies have shown that numbers of $CD3^{+}CD16^{+}CD56^{+}$ NKT-cells are also important, as a reduction in numbers is associated with disease progression and a higher risk of death in CLL patients (Bojarska-Junak *et al.* 2010). An understanding of the NK-cell defect may well prove to be clinically useful as pre-clinical studies have shown that CLL patient NK-cells can be expanded and show cytotoxicity against K562 cells, highlighting their potential as a source of cellular immunotherapy (Guven *et al.* 2003).

There is also evidence suggesting that monocytes and neutrophils are defective in CLL, with the latter having been shown to be deficient in lysozyme and myeloperoxidase, and to modulate CLL B-cell survival through altered secretion of TNF-superfamily proteins. (Zeya *et al.* 1979; Sawicka-Powierza *et al.* 2011)

## 1.5 Immunotherapy for CLL

Attempting to reconstitute the immune response in CLL is attractive for three reasons. First, the immune surveillance hypothesis suggests that in order to produce clinically detectable disease, the malignant CLL B cells must have evolved strategies of evading or suppressing the immune system, especially the anticancer effects of T cells. Therefore, successful immune reconstitution should lead to repair of antitumor immunity and durable clinical responses. Second, T cells provide "help" to B cells as part of a healthy immune system, by stimulating the B cells to proliferate, inducing B-cell antibody class switching, and promoting plasma cell differentiation. In CLL, there is evidence that T cells have been skewed to provide "help" for the malignant B cells, and therefore successful immune reconstitution should reduce the availability of T-cell–derived pro-CLL factors, leading to "starvation" of the CLL cells. Finally, even in the absence of any antitumor effects, immune reconstitution would benefit patients by enabling them to fight infections more effectively and would counteract the immune suppression induced by both the disease and current therapies. Strategies employing this approach should result in therapies that are more tolerable to vulnerable patients and show enhanced efficacy in the more challenging poor-risk subgroups.

### *1.5.1 Immunomodulatory aspects of cytotoxic chemotherapy*

Virtually all treatment for CLL is immunomodulatory in one sense or another. Treatments that are cytotoxic to the malignant B cells are invariably toxic to other leucocytes as well, both granulocytes and lymphocytes. One of the most important clinical aspects of cytotoxic chemotherapy is the degree to which it exacerbates the existing immunodeficiency. Indeed, the ability of a patient to tolerate the treatment is often an important predictor of overall prognosis. In addition to the effects of cytotoxic agents on the tumour cells, there is also the potential for differential toxicity to immune components of the pro-tumoural microenvironment. The purine analogue fludarabine is widely used for the treatment of CLL (Hallek *et al.* 2010). As T-cell depletion is a commonly encountered side effect of this agent, it can be hypothesized that fludarabine treatment will have a significant impact on the T-cell microenvironment. Analysis of the composition and function of the T cell pool in CLL patients after treatment with fludarabine and cyclophosphamide revealed a rapid and sustained reduction of circulating $CD4^+$ and $CD8^+$ T cells as expected. Surprisingly, T cells surviving this

combination had a more mature phenotype, and fludarabine-treated T cells were significantly more responsive to mitogenic stimulation than their untreated counterparts, and showed a shift towards Th1 cytokine secretion (Gassner *et al.* 2011). Furthermore, fludarabine therapy appears to have a particularly marked effect on $T_{regs}$, with both a reduction in the inhibitory function and a decrease in the frequency of this subset after treatment (Beyer *et al.* 2005). As both these studies were performed on PB samples it would be particularly interesting to examine the impact of fludarabine on CD4+ T cells in LNs and BM.

### *1.5.2 Improved NK-cell functionality with rituximab*

Rituximab is a humanized anti-CD20 monoclonal antibody (mAb) widely used in the treatment of B-cell malignancies and autoimmune diseases. The mechanism of action of this agent is not completely clear, although it appears to be due to a combination of several factors including antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), induction of apoptosis of $CD20^+$ B cells, inhibition of BCR-signalling, and regulation of the cell cycle (Maloney 2001). The efficacy of this agent in CLL is perhaps even more paradoxical, given the relatively low expression of its target antigen on CLL cells when compared with normal B cells and other malignant B cells. An important component of the mechanism of action of rituximab in CLL appears to be the enhancement of NK-cell functionality. Opsonisation of CLL cells by anti-CD20 antibodies such as rituximab, allows binding of the NK cell Fc receptor, FcγRIII (CD16), to the Fc region of the antibodies. Antibody binding to CD16 results in NK-cell activation, release of cytolytic granules, and consequent target cell apoptosis (Smyth *et al.* 2002). Rituximab has been shown to increase NK-cell degranulation as measured by the CD107a assay (Fischer *et al.* 2006). Interestingly, there is evidence that FcγR polymorphisms predict response to rituximab in lymphoma patients (Weng and Levy 2003). Third generation monoclonal anti-CD20 antibodies such as obinutuzumab (GA-101) and LFB-R603 that have higher binding affinities for this receptor have been shown to even more efficient at inducing NK cell degranulation (Bologna *et al.* 2011; Le Garff-Tavernier *et al.* 2011). *In vitro* experiments have demonstrated that NK-cell functionality can be further enhanced by co-culturing the NK cells with cytokines such as IL21 and IL15 (Eskelund *et al.* 2011; Moga *et al.* 2011). Opsonisation of the tumour cells by rituximab may also be important for the activity of other immune cells, as there is evidence that rituximab enhances macrophage killing of CLL cells by increasing phagocytosis mediated ADCC (Lefebvre *et al.* 2006).

### *1.5.3 "Eviction from the niche": the mobilising effect of BCR-signalling inhibitors*

There has been a great deal of recent interest in inhibitors of BCR-signalling for the treatment of CLL. BCR signalling is known to be crucial for B-cell proliferation and survival, and a number of major prognostic markers that are clinically useful in CLL, are associated with aberrations in BCR signalling. Absence of mutations in immunoglobulin variable (IgV) region genes and expression of ZAP70 have been shown to correlate with an inferior outcome, and are both associated with increased signalling through the BCR (Hamblin *et al.* 1999; Wiestner *et al.* 2003) This aberrant BCR-signalling has been thought to be antigen-dependent, but a recent publication has also suggested that BCRs from CLL patients induce antigen-independent cell-autonomous signalling, dependent on the heavy-chain complementarity-determining region (HCDR3) and an internal epitope of the BCR (Minden *et al.* 2012). A number of molecules that inhibit kinases downstream of the BCR have been developed, and are showing great promise in early phase clinical trials. An unexpected feature of some these agents is that there is a transient increase in the circulating lymphocytosis after initiation of treatment, associated with rapid reductions in LN size, consistent with mobilization of the tumour cells from the LNs into the blood. The PI3K inhibitor GS1101 (Idelalisib), the BTK inhibitor, ibrutinib (PCI-32765), and the mTOR inhibitor, everolimus, have all been found to induce this lymphocytosis during the first weeks of treatment (Burger *et al.* 2010; Flinn *et al.* 2010; Zent *et al.* 2010). As discussed above the CXCL12-CXCR4 axis is important for the homing of CLL cells to potential sanctuary sites in the BM and LNs. A small molecule inhibitor of CXCR4, plerixafor, has also shown clinical activity in CLL patients, again associated with mobilization of tumour cells into the circulation (Andritsos *et al.* 2010). It therefore appears likely that the some of the clinical activity of these novel therapies may be due to their ability to "evict" CLL cells from protective microenvironmental niches populated by NLCs, stromal cells and T cells (Hoellenriegel *et al.* 2010; Ponader *et al.* 2012).

### *1.5.4 CD40L based therapies*

A key feature of CLL cells is that they have impaired antigen presenting function, due to reduced expression of co-stimulatory molecules including CD80 and CD86.(Dazzi *et al.* 1995) CLL cell activation by ligation of CD40 can up-regulate these proteins, along with adhesion molecules such as ICAM1 (Ranheim and Kipps 1993; Yellin *et al.* 1994; Van den Hove *et al.* 1997). Under normal circumstances this activation signal is provided by T cells, which transiently express CD40L (CD154) after antigen engagement of their TCR ((Noelle *et al.* 1992). However, this critical axis is impaired in CLL, as the tumour cells appear to be able to directly down-modulate T-cell expression of CD40L (Cantwell *et al.* 1997). In light of this, a number of strategies have been developed to re-express CD40L in the tumour microenvironment, thereby improving CLL cell antigen presenting function and overcoming T-cell anergy.

One such strategy has been to use adenoviral vectors to transduce CLL cells to express CD40L. In addition to inducing expression of co-stimulatory and adhesion molecules on the transduced cells, increased expression of CD40L in the CLL microenvironment can activate other "bystander" CLL cells, even if they were not transfected. Further pre-clinical work showed that the transfected CLL cells become highly effective stimulators of mixed lymphocyte reactions, and induce generation of cytotoxic T cells specific for autologous unmodified CLL cells (Kato *et al.* 1998; Takahashi *et al.* 2001). Similar findings have been reported by culturing CLL cells on CD40L expressing feeder cells in place of transfection (Buhmann *et al.* 1999). Use of fibroblasts over-expressing CD40L and OX40L to transfer both these ligands to CLL cells, also resulted in the generation of tumour-reactive cytotoxic T lymphocytes (Biagi *et al.* 2005). There were some initial concerns that CD40-stimulated CLL cells may become more resistant to apoptosis induced by fludarabine, due to upregulation of the NFκB/Rel transcription factors.(Romano *et al.* 1998) Gene expression profiling performed on CD40-stimulated CLL cells also identified up-regulation of several genes involved in the suppression of apoptosis mediated by the TNF family of receptors and NFκB (Gricks *et al.* 2004). However further investigation showed that while CD40 stimulation enhanced the constitutive anti-apoptotic profile of the CLL cells, and these cells were not sensitive to CD95L, they remained good targets for cytotoxic T cells (Chu *et al.* 2000; Kater *et al.* 2004).

An early clinical trial infected the effects of infusions of autologous tumour cells that had been transduced *ex vivo* with murine CD40L, which was found to be more efficiently expressed than human CD40L. This treatment was well tolerated and the patients did show some PB and LN responses. However, some of the patients developed antibodies against the murine CD40L (Wierda *et al.* 2000). In light of this, a recombinant humanized CD40 binding protein, ISF35, was developed, and tested in a more recent phase I study. The infusions of transduced autologous tumour cells were again well tolerated, and were consistently followed by reductions in circulating lymphocyte counts and lymphadenopathy. The increased levels of this humanized CD40L was associated with induction of a pro-apoptotic state in the circulating CLL cells, with increased expression of pro-apoptotic molecules CD95, DR5, p73 and BCL-2 interacting domain (BID), and reduced levels of the anti-apoptotic molecule, MCL1. Significantly, these findings were also observed in patients with deletion of chromosome 17p (Wierda *et al.* 2010). There is a rationale for combining this approach with rituximab, as stimulation of CLL B cells by CD40L sensitizes them to rituximab-induced cell death (Jak *et al.* 2011). However of interest, evidence has emerged that there is significant heterogeneity in the responses of CLL B cells to CD40L stimulation. Patients with CLL B cells that were relatively unresponsive to CD40L showed a poor clinical outcome and shorter time to progression, which was felt to reflect less dependency on the microenvironment and higher autonomous proliferative and survival potential (Scielzo *et al.* 2011).

Other work has investigated whether there is a role for anti-CD40 mAbs in the treatment of CLL. Two therapeutic antibodies that target the CD40 antigen are in development, dacetuzumab (SGN-40) which acts as a partial agonist, and lucatumumab (HCD122) which blocks CD40 mediated signalling (Law *et al.* 2005; Luqman *et al.* 2008). Unfortunately, phase I trials using these agents have shown minimal single agent activity, but they warrant further investigation as part of combination therapies.(Furman *et al.* 2010; Byrd *et al.* 2012). Pre-clinical experiments have suggested that dacetuzumab treatment in combination with the immunomodulatory agent lenalidomide enhanced direct apoptosis and ADCC against primary CLL cells (Lapalombella *et al.* 2009). However, it is possible that this activity represents tumour cell opsonisation and the induction of NK-cell responses, as much as a direct biological effect of CD40 binding.

### *1.5.5 Vaccine therapy for CLL*

The observation that CLL cells maintain some residual B-cell function, which can be enhanced by appropriate stimulation, has led to attempts to induce an effective autologous immune response by vaccine therapy. A vital component of this approach is identifying a suitable tumour associated antigen (TAA), to which to induce an immune response. An ideal TAA should be only expressed on the tumour cells to avoid induction of auto-immunity, should be expressed on all of the tumour cells, and should be essential for tumour cell survival to prevent the emergence of antigen-negative variants and immune escape. The obvious major challenge to cancer vaccines is that the tumour cells are derived from "self", with the consequence that few TAAs have these characteristics. In light of this, the search for possible TAAs has focused on proteins that are either commonly mutated in the malignant cells or are aberrantly expressed. Serological identification by recombinant expression cloning (SEREX) has been used to detect such mutated proteins, and cytotoxic T-cell responses could be generated against these antigens (Krackhardt *et al.* 2002). A number of other TAAs have been identified in CLL, including survivin, fibromodulin, RHAMM (CD168), FMNL1, MDM2 and CD23 (**Table 1.6**) (Favaro *et al.* 2003; Schmidt *et al.* 2003; Mayr *et al.* 2005; Giannopoulos *et al.* 2006; Mayr *et al.* 2006; Bund *et al.* 2007). Of particular interest is the oncofetal surface antigen receptor tyrosine kinase-like orphan receptor 1 (ROR1), which has the advantage that it is selected expressed by malignant B cells, although it is also expressed by undifferentiated embryonic stem cells, and at low levels in adipose tissue (Hudecek *et al.* 2010). When 6 patients with CLL were infused with autologous CLL cells that had undergone adenoviral transfection with CD154, 3 of them (50%) subsequently generated antibodies against ROR1 (Fukuda *et al.* 2008). Furthermore, upregulation of CD154 by lenalidomide has also recently been shown to induce production of ROR1 tumour-specific antibodies, notably in a patient that had not received prior therapy with rituximab (Lapalombella *et al.* 2010).

**Table 1.6 Tumour-associated antigens in CLL**

| Type | Antigen | Expression patterns – potential for "on target, off organ" effects | Reference |
|---|---|---|---|
| **Normal/ malignant B-cell antigens** | Ig idiotype | Unique to an individual B-cell clone | (Janeway *et al.* 1975) |
| | CD19 | Expressed on follicular dendritic cells and normal B-cells | (Pesando *et al.* 1989) |
| | CD20 | Expressed on normal B-cells | (Cragg *et al.* 2005) |
| | CD23 | Expressed on mature B-cells, monocytes, follicular dendritic cells, and at low levels on T-cells, Langerhans cells, eosinophils and platelets | (Fournier *et al.* 1991) |
| | ROR1 | Expressed by malignant B-cells and undifferentiated embryonic stem cells, and at low levels in adipose tissue | (Baskar *et al.* 2008) |
| | Human Ly9 | Expressed on thymocytes and on mature T and B-cells | (Bund *et al.* 2006) |
| **Onco-fetal/ cancer-testis antigens** | RHAMM | Expressed in the thymus and testis; not expressed on healthy B-cells | (Giannopoulos *et al.* 2006) |
| | OFA-iLRP | Expressed in foetal tissues but not detectable on normal differentiated adult cells | (Siegel *et al.* 2003) |
| | SLLP1 | Expressed on spermatozoa; only expressed in the testis | (Wang *et al.* 2004) |
| | Survivin | Expressed during normal foetal development and in the adult thymus, testis, placenta, and growth hormone-stimulated hematopoietic stem and endothelial cells. | (Schmidt *et al.* 2003) |
| **Differential expression** | hTERT | Widely expressed, expression increased in 75% of CLL patients | (Kokhaei *et al.* 2007) |
| | Fibromodulin | Widely expressed, with the highest levels in articular cartilage, tendon, and ligaments | (Mayr *et al.* 2005) |
| | FMNL1 | Expressed in normal thymus, spleen and peripheral blood leucocytes; overexpressed in CLL | (Favaro *et al.* 2003) |
| | MDM2 | Widely expressed, over expressed in many malignancies | (Mayr *et al.* 2006) |
| | BAX | Widely expressed, enhanced proteasomal degradation in CLL generates expression of degradation peptides | (Nunes *et al.* 2011) |

A particular feature of B-cell malignancies is that there is a further type of TAA in the form of the immunoglobulin idiotype (Janeway *et al.* 1975). The surface immunoglobulin expressed by the malignant B-cells is unique to each clone, and thus represents an attractive target for vaccine strategies. Using a bioinformatics approach, a number of human immunoglobulin derived peptides were identified that were capable of inducing cytotoxic T-cells. Furthermore, autologous cytotoxic T cells specific for these peptides were able to kill primary malignant CLL cells. One of the problems with this method is that the very uniqueness of the idiotype means that any potential vaccine would be have to individualized to each patient. However, peptides generated from the framework regions of the immunoglobulin molecule, regions that show less variability between patients, were immunogenic, allowing for a single such peptide to be used in a

number of patients (Trojan *et al.* 2000). A second problem with idiotype vaccination strategies is that the immunoglobulin idiotype is only weakly immunogenic and often only generating humoral responses (Kwak *et al.* 1992; Hsu *et al.* 1997). A solution to this is the use of heteroclitic peptides, where MHC-binding amino acid residues are modified to bind MHC more tightly, while leaving the T-cell recognition residues intact, inducing more potent immune responses (Harig *et al.* 2001).

In the normal immune system, the most potent antigen presenting cells are dendritic cells (DCs). This property can be exploited by manipulating DCs to take up antigen *in vitro,* before presenting it to T cells causing their activation and proliferation. There have been a number of clinical studies that have investigated the use of DCs in CLL. In one such study, DCs were generated from unrelated donors, before being pulsed *ex vivo* with patient CLL cell lysate or apoptotic bodies. They were subsequently administered to patients with early-stage CLL, a subset of whom showed clinical responses. These investigators then looked at autologous DCs pulsed with CLL cell lysates, and again a subset of patients showed responses, that were associated with an increase in T cells specific for RHAMM/CD168 and fibromodulin (Hus *et al.* 2005; Hus *et al.* 2008). A further study used oxidizing radiation to augment the immunogenicity of the CLL cells by improving their antigen presenting function, resulting in enhanced T-cell anti-tumour activity and clinical responses. (Spaner *et al.* 2005).

### *1.5.6 Immune checkpoint blockade in CLL*

Suppression of anti-tumour immunity by inhibitory pathways appears to be an important mechanism underlying the failure of immune responses in CLL. The tumour cells have been demonstrated to express high levels of inhibitory ligands, which inactivate immune cells in the microenvironment via their increased expression of receptors for these ligands. An example of one such set of interactions is the PD1:PDL1 axis, which has been shown to be functionally important in suppressing immunological synapse formation between CLL cells and autologous T cells (Ramsay *et al.* 2012; Brusa *et al.* 2013). This axis had previously been demonstrated to suppress T-cell function in chronic viral infections in both mice and humans (Barber *et al.* 2006; Day *et al.* 2006). These same studies also showed that blocking PD1:PDL1 interactions could restore T-cell effector function, which could be further enhanced by simultaneous blockade of other inhibitory receptors such as CTLA4, LAG3 and TIM3 (Blackburn *et al.* 2009; Nakamoto *et al.* 2009; Jin *et al.* 2010). These findings have led to considerable interest in the potential for blockade of these "immune checkpoints" to enhance anti-viral and anti-tumour immunity in chronic viral infections and cancers (Pardoll 2012). Initial work highlighted the potential for blockade of CTLA4 to induce clinical responses in patients with advanced melanoma (Hodi *et al.* 2010). Subsequent studies have also investigated the effect of blocking the PD1:PDL1 axis. A recent phase 1 study of an anti-PD1 monoclonal antibody (Nivolumab; BMS-936558) in patients with non-small cell lung cancer, melanoma, or renal cell cancer, showed ORRs of 18-28% dependent on the cancer type (Topalian *et al.* 2012). These response rates are very encouraging given the 10-15% "ceiling" of durable tumour response rates seen with trials of other immunotherapeutic approaches in solid cancers over the last 30 years (Ribas 2012). A further phase 1 trial of an anti-PDL1 antibody (BMS-936559) in a variety of advanced solid cancers also showed efficacy, with response rates of 6-17%, again dependent on the cancer type (Brahmer *et al.* 2012). Clinical trials of these agents in haematological malignancies have been notably absent, despite the fact that these cancers are generally more "immunosensitive" with the potential for higher response rates. A single published phase 1 dose-escalation study of another anti-PD1 antibody (CT-011; Pidilizumab) showed evidence of response in 6 out 18 patients. Of the three CLL patients that were enrolled, two showed evidence of stable disease, on the second and third dose levels (Berger *et al.* 2008). Given the pre-clinical data highlighting the significance of the PD1:PDL1 axis in suppressing T-cell function in CLL, there is a strong rationale for clinical assessment of immune checkpoint blockade in this disease.

### *1.5.7 Adoptive T cell therapies*

Currently, allogeneic haematopoietic stem cell transplantation remains the only curative option for CLL. Critical to its activity is the graft-versus-leukaemia (GVL) effect, where the transplanted haematopoietic stem cells differentiate into effector cells that mount an anti-tumour immune response. This effect is known to be primarily T-cell mediated, and is probably due to a combination of improved T-cell function and the presence of allogeneic major histocompatibility complex (MHC) molecules (Kolb 2008). The major drawback of myeloablative allogeneic transplantation is that it is associated with unacceptably high rates of non-relapse mortality, that can approach 50% even in younger patients (Michallet *et al.* 1996). This is due to combination of toxicity from the conditioning regimens, the prolonged period of immune-suppression during engraftment, and graft-versus-host disease (GVHD). One way of attempting to reduce non-relapse mortality is to use reduced intensity conditioning (RIC) to exploit the GVL effect while avoiding the significant morbidity and mortality associated with myeloablative conditioning. RIC regimens are associated with decreased mortality rates, but GVHD remains a significant problem (Dreger *et al.* 2003; Schetelig *et al.* 2003; Sorror *et al.* 2005). Therefore for the majority of patients, who are above the age of 65 years, allogeneic hematopoietic stem cell transplantation is not a reasonable option.

One approach to circumventing the problem of GVHD is the adoptive transfer of T cells with specificity for tumour antigens. By choosing T cells with specificity for only tumour cells, it should be possible to produce immune mediated anti-tumour responses without the corresponding GVHD. There are two main strategies for generating tumour specific T cells. The first involves the gene transfer of T-cell receptors (TCRs) with known specificity into autologous or allogeneic T cells which are then expanded *in vitro* and infused into patients. This approach has had some successes, most notably in melanoma and the use of Epstein-Barr virus specific T-cells to treat post-transplant lymphoproliferative disorders (Rooney *et al.* 1998; Morgan *et al.* 2006; Rosenberg *et al.* 2011). However a potential risk of this approach is that the α or β subunit of the transgenic TCR could misassociate with the α or β subunit of the endogenous TCR, resulting in an autoreactive T-cell. Furthermore, a particular drawback to widespread clinical use is that the recognition of the tumour antigens is MHC-restricted, and therefore the use of these T cells needs to be individualized on a patient-by-patient basis according to their MHC type. A second strategy involves the use of the single chain variable fragment from an antibody molecule fused with an internal signalling domain

such as CD3ζ, to form a chimeric antigen receptor (CAR)(June *et al.* 2009). There are two important advantages to this approach. Firstly, it eliminates MHC restriction enabling the same CAR to be used for several different patients. Secondly, the use of an antibody receptor means that potential targets can be increased to include a wide range of surface proteins, sugars and lipids (Cartellieri *et al.* 2010)(**Figure 1.9**).

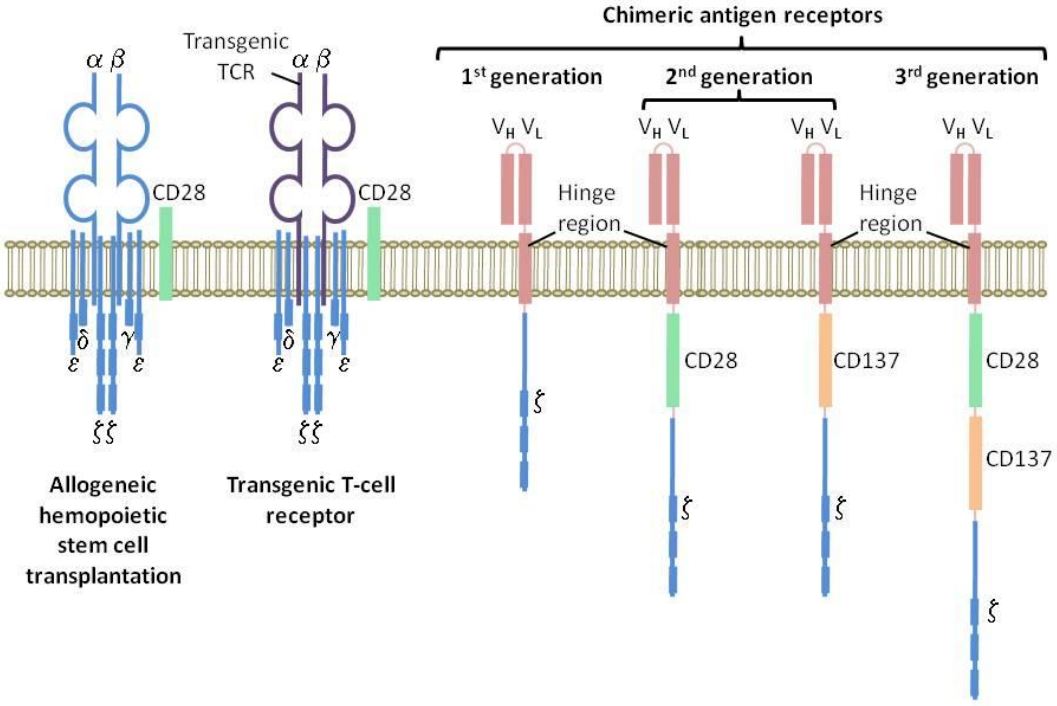



**Figure 1.9 Adoptive T cell therapies in CLL**

T-cell mediated therapies include the adoptive transfer of allogeneic T cells as part of haematopoietic stem cell transplantation, the transfer of allogeneic or autologous T cells with a transgenic T-cell receptor, and the transfer of T cells with chimeric antigen receptors (CARs). Early studies used CAR T cells without a co-stimulatory domain (1st generation). Current trials are using 2nd-generation CAR T cells, with either a CD28 or CD137 (4-1BB) co-stimulatory domain. Preclinical studies are investigating the combination of co-stimulatory domains (3rd generation).

The target of these CARs must be carefully selected to avoid "on-target, off-organ" effects, which potentially can occur when the antigen is also expressed on non-malignant tissues. In the context of CLL, particularly attractive targets are CD19, CD20, CD23, and ROR1. CLL B cells express high levels of CD19, in contrast to the relatively reduced expression of CD20. A disadvantage of targeting these molecules is that they are also expressed by normal B cells, so CAR T cells targeting them will also eliminate normal B cells, causing persistently impaired humoral immunity and exacerbating the

immunodeficiency already present in CLL (Koehler *et al.* 2012). Anti-ROR1 CAR $CD8^+$ T cells that recognize autologous CLL B cells have been successfully generated from patients with CLL. ROR1 has the advantage of being selectively expressed by malignant B cells, although it is also expressed by undifferentiated embryonic stem cells and (at low levels) in adipose tissue (Hudecek *et al.* 2010). Similarly, anti-CD23 CAR T cells generated from CLL patients have shown cytotoxicity against autologous and allogeneic CLL cells and also have shown an *in vivo* antitumor effect in a xenograft murine model (Giordano Attianese *et al.* 2011).

A number of phase I/II clinical trials are underway using anti-CD19 CAR T cells for the treatment of B cell malignancies (Koehler *et al.* 2012). One group has treated 8 patients with CLL in 2 cohorts. The first cohort of 3 patients did not receive any conditioning, and did not show any objective responses. They went on to give the next patient lymphodepleting conditioning with cyclophosphamide as part of the trial design. Unfortunately this patient rapidly developed hypotension, respiratory distress and renal failure, and died within 48 hours of infusion of the T-cells, highlighting the risks associated with this therapy (Brentjens *et al.* 2010). They have gone on to treat a further 4 patients with cyclophosphamide conditioning and a reduced dose of T cells, with 3 out of 4 of the patients showing disease stabilization or LN responses (Brentjens *et al.* 2011). A second group has investigated the use of anti-CD19 CAR T cells in 8 patients with progressive B-cell malignancies, including 4 patients with CLL. These patients received conditioning with cyclophosphamide and fludarabine, followed by infusion of anti-CD19 CAR T cells. They also received a post infusion course of interleukin-2 to enhance *in vivo* T-cell expansion. Six of the 8 patients obtained objective remissions: of the 4 CLL patients 1 had a complete response, 2 had partial responses, with 1 having stable disease. This trial also investigated some of the potential adverse effects associated with the use of CAR T cells. Four out of the 8 patients had increased serum levels of IFNγ and TNFα, which correlated with the severity of acute toxicities. It is likely that the CAR T cells were a source of these inflammatory cytokines because T cells isolated from the patients after treatment produced IFNγ and TNFα *ex vivo* in a CD19-specific manner. These trials have underscored the importance of the conditioning regimen in promoting T-cell engraftment and activation. As discussed above, it may be vital to use conditioning regimens that deplete the microenvironment of pro-tumoural cell types such as regulatory T cells or immature dendritic cells. One important aspect of forthcoming clinical trials in this area will be the determination of

which agents to use in the conditioning regimen, analogous to the impact of conditioning in allogeneic transplantation.

A key finding in pre-clinical studies of CAR T cells was that their efficacy could be significantly improved by the addition of a co-stimulatory domain such as CD28 (Maher *et al.* 2002). It is becoming apparent that the choice of co-stimulation can have significant impact on the clinical success of CAR T cell-based therapies. Many of the CAR T cells currently under investigation in clinical trials have incorporated the use of the CD28 co-stimulatory domain, including the two trials discussed above (Koehler *et al.* 2012). However, in a murine model of primary human pre-B-cell acute lymphoblastic leukaemia, human T cells expressing anti-CD19 CARs containing the co-stimulatory domain CD137 (4-1BB) were significantly more effective and showed longer survival than cells expressing CARs containing the CD28 domain (Milone *et al.* 2009). Furthermore, pre-clinical data suggests that CD137 is less likely to trigger IL-2 and TNF-α secretion, and hence induction of a "cytokine storm" and differentiation of regulatory T-cells. In light of this, other investigators have trialled the use of anti-CD19 CAR T-cells incorporating a CD137 co-stimulatory domain. A recent report documented a case of a heavily pre-treated patient with refractory CLL who entered a complete remission after the adoptive transfer of anti-CD19 CAR T-cells. A significant feature of this case was that these cells were still detectable at 6 months post infusion, and had started to express molecules associated with a central memory phenotype, which is known to be important in maintaining robust and persistent anti-tumour immune responses (Porter *et al.* 2011). A further 2 cases have been reported one of whom also achieved complete remission after being heavy pre-treated. While this is encouraging, there remain many unresolved questions. 169 days after infusion the CAR T cells had high expression of CD45RA, PD1 and CD57, which may reflect the extensive replication history of these cells leading to T-cell exhaustion and incipient loss of function (Kalos *et al.* 2011; Wherry 2011). This could lead to treatment failure in the longer term, and necessitate further infusions of these T cells to maintain clinical responses. Furthermore, the risk associated with profound long-term B-cell lymphopoenia and hypogammaglobulinaemia is still unknown, and could result in increased susceptibility to infections analogous to Bruton's X-linked agammaglobulaemia.

As discussed above, other issues remain, such as the best time to use these cells, the type of conditioning and costimulatory domain, and which antigen is best to target. The malignant cells may develop the ability to downregulate CD19, necessitating the use of CAR T cells targeting alterative tumour antigens such as CD23 and ROR1, or the use of combinations of CAR T cells specific for a range of antigens.(Hudecek *et al.* 2010; Giordano Attianese *et al.* 2011) However, the potential of CAR T cells, and indeed the possibility of engineering other cell types with CARs such as NK and NKT cells, means this remains an exciting area of research (Boissel *et al.* 2009).

**Table 1.7 Summary of Immunotherapeutic approaches in CLL**

| **Mechanism** | **Examples** |
|---|---|
| CLL cell mobilization from sanctuary sites/ CXCR4-CXCL12 blockade | - Plerixafor<br>- CAL-101<br>- PCI-32765<br>- Everolimus |
| Repair of CLL B-cell antigen presenting function | - CD40L gene therapy<br>- Lenalidomide |
| Upregulation of CD40L | - CD40L gene therapy<br>- Lenalidomide |
| T-cell activation and repair | - Lenalidomide<br>- Interleukin-2<br>- Immune checkpoint blockade |
| Enhanced T-cell co-stimulation | - Lenalidomide<br>- Immune checkpoint blockade<br>- $2^{nd}/3^{rd}$ generation chimeric antigen receptor T-cells |
| Targeting tumour-associated antigens | - Peptide vaccines<br>- Dendritic cell vaccines<br>- Transgenic TCR T-cells<br>- Chimeric antigen receptor T-cells |
| Adoptive T-cell therapy | - Allogeneic hematopoietic stem cell transplantation/donor lymphocyte infusions<br>- Transgenic TCR T-cells<br>- Chimeric antigen receptor T-cells |

## 1.6 Lenalidomide

### *1.6.1* History and Development: Immunomodulatory aspects

Lenalidomide (Revlimid, CC-5013) is a second-generation derivative of thalidomide with anti-tumour, anti-angiogeneic and immunomodulatory activity. Its parent compound, thalidomide, was developed in 1954 by CIBA, and was initially used as a sedative, tranquiliser, and anti-emetic for the treatment of morning sickness (D'Amato *et al.* 1994). Unfortunately, thalidomide was found to have potent and tragic teratogenic effects, and was withdrawn from the market (Lenz 1988). Despite this, it was noted to be an effective treatment for erythema nodosum leprosum (ENL), an acute inflammatory manifestation of lepromatous leprosy, and remained in use for this indication (Sheskin 1965). In the 1980s it became apparent that thalidomide was useful in the treatment of other inflammatory diseases, including rheumatoid arthritis, Behcet's disease, systemic lupus erythematosus, and even GVHD (Gutierrez-Rodriguez 1984; Hamza 1986; Lim *et al.* 1988; Atra and Sato 1993). This anti-inflammatory activity was subsequently linked to inhibition of TNFα production, as patients with ENL who responded to thalidomide were noted to have rapid reductions in serum concentrations of TNFα, and thalidomide was observed to suppress production of TNFα by lipopolysaccharide (LPS)-stimulated monocytes *in vitro* (Sampaio *et al.* 1991; Sampaio *et al.* 1993).

As a result of these findings, investigators at Celgene proceeded to synthesise several analogues of thalidomide which were optimised for their ability to control TNFα production (Corral *et al.* 1996; Muller *et al.* 1996). Second generation immunomodulatory drugs (IMiD) including lenalidomide and pomalidomide were found to be up to 50,000-fold more potent than thalidomide at inhibiting TNFα production by PBMC *in vitro*. Interestingly, the effect of these agents was dependent on the type of stimulation applied to the PBMCs, as while they inhibited IL1β, IL12, and IL6 production/increased IL10 production from LPS-stimulated PBMCs, they increased TNF and IL12 production/decreased IL10 production by anti-CD3 stimulated PBMCs. Furthermore, these agents were noted to have marked co-stimulatory effects on anti-CD3 stimulated T cells, increasing T-cell proliferation and production of IL2 and IFNγ (Haslett *et al.* 1998; Corral *et al.* 1999).

The exact mechanism by which IMiDs such as lenalidomide and pomalidomide exert these effects is unclear. Enhanced activity of the transcription factor AP1 has been shown to play a role, as increased IL2 production by Jurkat T cells correlated with an increase in IL2 promoter activity and IL2 mRNA levels that could be eliminated by targeted mutations of the proximal AP1 binding site (Schafer *et al.* 2003). Further work demonstrated that pomalidomide treatment could enhance the DNA-binding activity of AP1, suggesting that the co-stimulatory effect of pomalidomide on T cells was due to enhanced binding of AP1 to its binding site in the proximal region of the IL2 promoter (Payvandi *et al.* 2005). Interestingly, the DNA-binding-enhancing effect of pomalidomide was *not* observed with other transcription factors such as NFκB, NFAT or OCT1. Furthermore, the increased activity of AP1 was *not* associated with many of the upstream signalling events that are classically involved in T cell activation, such as calcium flux, and NFAT, ERK, JNK, Jun or p38 MAPK phosphorylation. Subsequent work instead suggested that activation of PKCθ was important, as the co-stimulatory effect of pomalidomide could be blocked by two inhibitors of PKCθ, Rottlerin and G06976, and pomalidomide was observed to directly enhance the activity of PKCθ in stimulated T cells in an *in vitro* kinase assay. In contrast, other investigators have suggested that the co-stimulatory effect of IMiDs is mediated by the B7-CD28 pathway. In this study, lenalidomide was able to partially overcome the inhibitory effect of CTLA4 by triggering tyrosine phosphorylation of the intracellular domain of CD28 in T cells, with downstream activation of the NFκB pathway (LeBlanc *et al.* 2004). This conflicting data could be attributed to the different methods used for T-cell stimulation, with calcium channel activation by PMA/Ionomycin being used in the initial reports highlighting the importance of AP1 activation, and TCR-stimulation by anti-CD3 being used in the report suggesting involvement of the B7-CD28 pathway (Schafer *et al.* 2003; LeBlanc *et al.* 2004; Payvandi *et al.* 2005). However, this does highlight the controversy that still surrounds the exact mechanism of action of lenalidomide's effect on T cells (McDaniel *et al.* 2012). Indeed, another report has suggested that the co-stimulatory effect of IMiDs relies on an entirely different mechanism. A recent investigation into the effects of IMiDs reduced on immune cells in myeloma patients, suggested that these agents were able to downregulate both T- and NK-cell expression of Suppressor of Cytokine Signalling-1 (SOCS1), an important negative regulator of cytokine signalling, thereby leading to enhanced cytokine production (Gorgun *et al.* 2010).

In addition to their co-stimulatory effects, IMiDs have also been demonstrated to enhance T-cell cytotoxic function. Initial work demonstrated the ability of thalidomide to enhance the capacity of purified $CD8^+$ cells to lyse allogeneic monocytes after priming by HLA-mismatched dendritic cells (Haslett *et al.* 1998). Further work by the same group showed that thalidomide and lenalidomide were able to enhance virus specific CD8+ T-cell cytokine production and cytotoxic activity in both healthy donors and patients chronically co-infected with HIV and CMV. The mechanistic explanation for the ability of IMiDs to enhance cytotoxic function appears to lie in their capacity for cytoskeletal activation, which is a critical regulator of T-cell function. This area has been of particular interest to my group after they showed that the functional defect of T cells in CLL was associated with alterations in the expression of cytoskeletal genes and defective immunological synapse formation. My supervisors subsequently demonstrated that treatment of both autologous T cells and CLL cells with lenalidomide resulted in repair of immunological synapse formation and improvement in $CD8^+$ T-cell cytotoxic responses suggesting that this may be a key component of this agent's activity in CLL (Ramsay *et al.* 2008). Further work by my group has demonstrated that lenalidomide was able to block CLL-cell induced inhibition of the RhoGTPases, RhoA, Cdc42, and Rac1, in healthy T cells (Ramsay *et al.* 2012). This effect appears to be due to a combination of an activatory effect of lenalidomide on these RhoGTPases, and to down-regulation of inhibitory signalling axes between CLL cells and T cells (Ramsay *et al.* 2013). Other investigators have noticed similar effects: in particular, it has been shown that both lenalidomide and pomalidomide can activate the cytoskeletal regulators Rac1 and RhoA in T cells and CLL cells (Xu *et al.* 2009; Troeger *et al.* 2012). Further work implicated these RhoGTPases in the regulation of IL2 production, as RhoA was essential for pomalidomide-induced IL2 expression in T cells, which could be blocked by treatment with a ROCK1 inhibitor. Interestingly, otherwise unrelated work has provided evidence that RhoA is able to stimulate expression of members of the AP1 family of transcription factors, providing further evidence that could potentially link the cytoskeletal and co-stimulatory effects of IMiDs (Marinissen *et al.* 2004).

Lenalidomide also has effects on other non-malignant lymphocyte subsets. It has been shown to significantly inhibit the suppressor function and generation of $T_{regs}$ *in vitro*, and was found to reduce numbers of $T_{regs}$ in PB of CLL patients.(Galustian *et al.* 2009; Idler *et al.* 2009) There is also evidence to suggest that lenalidomide is able to alter the homeostatic regulation of T-cell subsets. Correlative immune studies performed on

patients undergoing treatment for MDS showed that patients with an erythroid response to lenalidomide had a greater increase in naive and central memory T-cell subsets compared to non-responders, with a concurrent decrease in effector memory subsets (McDaniel *et al.* 2012). A similar increase central memory T cells was observed in a study of myeloma patients that had received lenalidomide in combination with a pneumococcal vaccine (Noonan *et al.* 2012). Lenalidomide has also been shown to have comparable effects on NK cells, enhancing their proliferative and cytotoxic capacity. IL2 again appears to be critical, as increased NK-cell production of IFNγ, increased expression of CD69, and enhanced cytolytic activity, were all dependent on pomalidomide-induced production of this cytokine (Payvandi *et al.* 2005). Again, as in T cells, lenalidomide has been shown to immunological synapse formation between NK cells and target cells (Gaidarova *et al.* 2009). Lenalidomide has also been shown to downregulate the expression of inhibitory receptors on NK cells, and to enhance antigen specific expansion of NK T cells (Chang *et al.* 2006; Dauguet *et al.* 2010).

A recent paper has identified cereblon (CRBN) as a primary target of thalidomide teratogenicity. Thalidomide binds to CRBN inhibiting the activity of a E3 ubiquitin ligase complex compromised of CRBN, DNA damage binding protein-1 (DDB1), cullin 4A, and ring-box protein-1 (RBX1, also known as Roc1). This was shown to lead to the down-regulation of fibroblast growth factor genes and teratogenic effects in zebrafish and chick models (Ito *et al.* 2010). CRBN is also implicated in other pathways, as it binds to a calcium-activated potassium channel in the brain, a chloride channel in the retina, and to AMP kinase, a master sensor of energy balance in eukaryotic cells (Jo *et al.* 2005; Hohberger and Enz 2009; Lee *et al.* 2011). In addition, a nonsense R419X mutation of CRBN is associated with autosomal recessive non-syndromic mental retardation (Higgins *et al.* 2008).

Further work has shown that CRBN-binding is also a critical component of the activity of lenalidomide and pomalidomide, as its expression was required for the anti-myeloma effect of these agents (Zhu *et al.* 2011). Although CRBN depletion was initially cytotoxic to human myeloma, surviving cells with stable CRBN depletion became resistant to IMiDs, but not to other agents such as dexamethasone, bortezomib, or melphalan. Gene expression changes induced by lenalidomide where suppressed by CRBN knockdown, demonstrating that CRBN is required for the downstream activity of this agent. Interestingly, these investigators noted that CRBN was required for

lenalidomide-induced down-regulation of interferon regulatory factor 4 (IRF4), a transcription factor that is known to be critical for myeloma cell survival (Shaffer *et al.* 2008). Subsequent work has focused on the ability of IMiDs to modulate the activity of the CRBN-DDB1-Cul4A-Roc1 E3 ubiquitin ligase complex. Under normal circumstances this complex adds ubiquitin either to CRBN itself (auto-ubiquitination) or to a variety of substrate proteins. The IMiDs are all able to inhibit the auto-ubiquitination of CRBN, but are also thought to be able to modulate the specificity of this ubiquitin ligase complex for other substrate proteins, inhibiting ubiquitination of some proteins and increasing ubiquitination of others. Importantly, evidence is emerging that CRBN is critical for the effect of lenalidomide and pomalidomide on T cell activation, as siRNA knockdown of T-cell CRBN blocked the increase in production of IL-2 and TNF-α induced by these agents (Lopez-Girona *et al.* 2012) Furthermore, recent work has suggested that this is specifically due to an effect on the CRBN-DDB1-Cul4A-Roc1 E3 ubiquitin ligase complex, rather than another of the potential pathways (Kronke *et al.* 2012). The current understanding of the mechanism of action of lenalidomide on T cells is summarised in **Figure 1.10**.

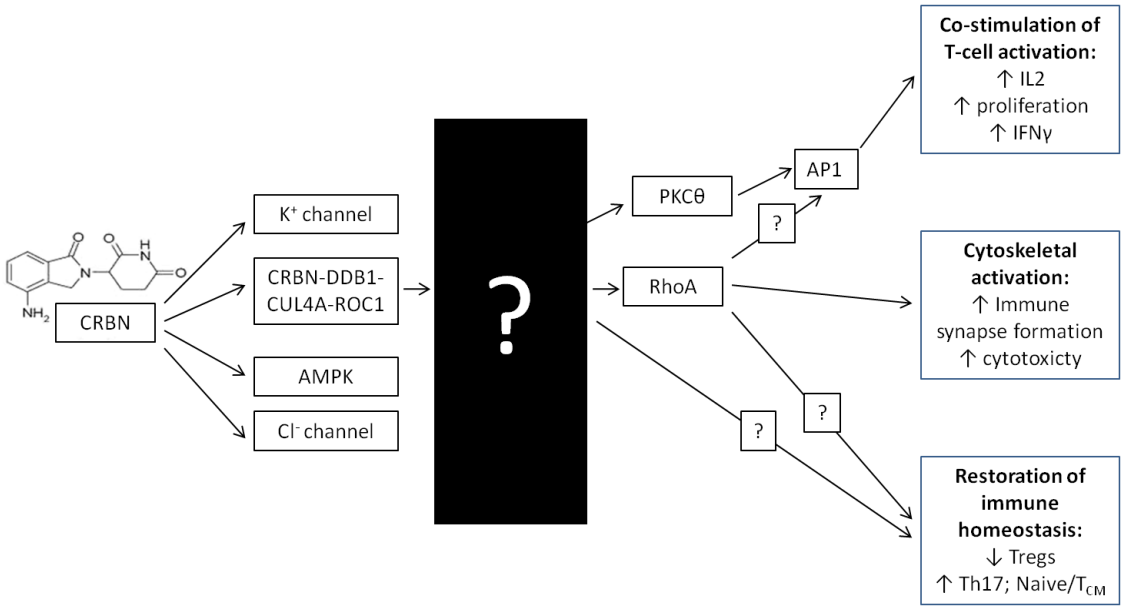



**Figure 1.10 The effect of lenalidomide on T cells**

Lenalidomide binds CRBN, which results in several potential effects, including modulation of the E3 ubiquitin ligase activity of a complex of CRBN with DDB1, CUL4A, and ROC1. The subsequent downstream events remain unclear, but emerging evidence suggests that this ubiquitin ligase activity is important for the co-stimulatory effects. Lenalidomide activates PKCθ and RhoGTPases such as RhoA, which result in enhanced co-stimulation, cytoskeletal activation and restoration of T cell homeostasis.

### *1.6.2* The mechanism of action of lenalidomide in CLL

Lenalidomide has been tested in several phase I and phase II clinical trials in CLL. A particularly interesting aspect of the clinical activity of lenalidomide in CLL is the tumour flare (TF) reaction. This is manifested by acute and painful swelling of involved lymph nodes, hepatosplenomegaly, rash, and fever, which usually occurs 8 – 72 hours after initial drug administration (Andritsos *et al.* 2008; Chanan-Khan and Cheson 2008; Aue *et al.* 2009) It can be effectively treated with both steroids and non-steroidal anti-inflammatories: in later trials these agents were used prophylactically, which led to reports of a delayed TF reaction developing at the time of steroid taper (Andritsos *et al.* 2008). Repeat TF symptoms were also reported to develop in subsequent cycles on resuming lenalidomide after the week off therapy where the drug was given for 21 days out of a 28 day cycle (Chen *et al.* 2010). The clinical manifestations are associated with features of immune activation, with upregulation of CD40, CD80, and CD86 on CLL cells, upregulation of CD69 on T cells, and elevated serum levels of cytokines and chemokines including IL6, IL8, TNFα, CCL2, CCL3, CCL4, and the soluble receptor IL1Rα (Andritsos *et al.* 2008; Aue *et al.* 2009). There has been some suggestion that the presence of a tumour flare reaction correlates with clinical outcome, highlighting the fact that the mechanisms underlying this phenomenon may also account for lenalidomide's anti-tumour effect (Chanan-Khan *et al.* 2010; Badoux *et al.* 2011). However this correlation has not been observed in all studies (**Table 1.8**). This could be due to the early experience with lenalidomide, that suggested that TF reactions could be potentially dose limiting. As a consequence of this, subsequent clinical trials sought to minimise the risk of TF by adjustments to lenalidomide dosing and scheduling, and by the use of prophylaxis. There also appears to be a confounding effect of previous therapy, given the observation of a higher incidence of TF in treatment naive patients, who potentially have more robust immune systems that are more sensitive to the immunostimulatory effects of lenalidomide.

Therefore, the clinical activity of lenalidomide in CLL appears to be due to combination of activation of the CLL cells themselves, in combination with activation of components of the immune microenvironment, particularly T and NK cells. The importance of lenalidomide-induced immune cell activation is highlighted by observations that lenalidomide is not directly toxic to CLL cells *in vitro* (Chanan-Khan and Porter 2006; Chanan-Khan *et al.* 2011). This is in contrast to the situation in myeloma, where lenalidomide has a direct toxic effect ((Chang and Stewart 2011)). Notably, the toxic

**Table 1.8 The tumour flare reaction in clinical studies of lenalidomide in CLL**

| Clinical trial | Lenalidomide dose | Tumour flare | Predicts better response? |
|---|---|---|---|
| (Chanan-Khan *et al.* 2006) | 25mg/day, then 5 mg, with 5mg escalations to 25mg/day (21/28 days) | 58% of patients (8% grade III/IV) | Yes |
| (Ferrajoli *et al.* 2008) | 10mg/day, with 5mg escalations to 25mg/day (continuous) | 12% of courses (2% grade III/IV) 30% of patients | No |
| (Aue *et al.* 2009) | 20mg/day for first 10 patients 10mg/day for next 8 patients | 83% of patients | *In vitro* response of CLL cells predicts clearance from PB |
| (Andritsos *et al.* 2008) | 25mg/day (21/28 days) | 75% | Not analysed |
| (Chen *et al.* 2010) | 2.5mg/day, 5mg, 10mg (21/28 days) | 88% of patients (0% grade III/IV) | No |
| (Badoux *et al.* 2011) | 5mg/day, with 5mg escalations to 25mg/day (continuous) | 52% of patients (0% grade III/IV) | No (but p = 0.058) BUT: TFR predicts for longer PFS |
| (Badoux *et al.* 2013) | 10mg/day (continuous) | 27% of patients (0% grade III/IV) | Not analysed |

effect in myeloma is mediated by binding of lenalidomide to CRBN, and clinical responses in myeloma do correlate with CRBN levels, with levels falling in resistant disease (Zhu *et al.* 2011). This is not the case in CLL, where baseline expression of CRBN in the tumour cells does not appear to predict for response (Chen *et al.* 2012). Instead the immune activating effects of lenalidomide are more important in CLL, with CLL-cell and T/NK-cell activation resulting in increased tumour-immune cell interactions and enhanced anti-tumour immune responses. Indeed, NK-cell activation appears to be a predictor for clinical response to lenalidomide in CLL. The presence of higher numbers of NK-cells pre-treatment, a greater capacity for NK-cell proliferation *in vitro*, and higher baseline expression of NK-cell activating ligands MIC-A/B on CLL cells, all predicted for better outcome in a clinical trial (Chanan-Khan *et al.* 2011; Chanan-Khan *et al.* 2011).

A number of studies have investigated the effect of lenalidomide on CLL cells, aiming to account for its ability to increase expression of co-stimulatory and activation markers such as CD40, CD80, CD83 and CD86. One group reported that *in vitro* treatment of CLL cells with lenalidomide was able to trigger MAPK/ERK signalling, increasing

levels of p-ERK (Chanan-Khan *et al.* 2011). Interestingly, this conflicts with previous reports in T cells, where lenalidomide/pomalidomide treatment was not found to have any effect on ERK signalling (Schafer *et al.* 2003; Payvandi *et al.* 2005). However, these agents were initially reported to have paradoxical effects dependent on the type of cell being stimulated, and the nature of stimulation (Corral *et al.* 1999), so it is possible that the mechanism of action of IMiDs varies with the target cell. Other studies have implicated a role for the PI3-kinase pathway in lenalidomide-induced CLL-cell activation (Herman *et al.* 2011). The combination of PI3-kinase activation and induction of NFκB and NFAT signalling has been showed to result in enhanced transcription and stabilisation of CD154 mRNA. The leads to expression of CD154 (CD40L) on CLL cells, which upregulate BID, DR5 and p73, and become sensitised to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. The upregulation of CD40L also promotes co-stimulatory activation of normal B cells, resulting in the development of polyclonal antibodies, including antibodies directed at known tumour-associated antigens such as ROR1 (Lapalombella *et al.* 2010). This data highlights the complex interplay of nature of cellular interactions that are induced by lenalidomide treatment in CLL.

### *1.6.3* Clinical experience with lenalidomide in CLL

At the time of writing, there have been eight major published clinical studies of lenalidomide in CLL (summarised in **Table 1.9**). Initial findings in pre-treated patients were encouraging, with an ORR of 58%, but this efficacy was not reproduced in two further studies in relapsed/refractory patients (Chanan-Khan *et al.* 2006; Ferrajoli *et al.* 2008; Wendtner *et al.* 2012). This may be due to the dose of lenalidomide used. The initial study used a starting dose of 25mg/day in the majority of patients, which is the standard target dose used in the treatment of multiple myeloma (where it is relatively well tolerated). However, as discussed above, one of the clinical features of the use of lenalidomide in CLL is a tumour flare reaction, and concerns about this phenomenon led to a reduction in the doses used in subsequent studies. Further trials using single-agent lenalidomide in previously untreated patients have been more successful. Indeed, lenalidomide has been demonstrated to have significant clinical activity, with an ORR of 72%, which compares well to single-agent response rates seen with other commonly used therapies such as fludarabine, alemtuzumab, bendamustine, and chlorambucil (Chen *et al.* 2010). Importantly, a recent phase II trial has demonstrated similar activity in elderly ($\geq$ 65 years) patients, with lenalidomide being well tolerated when used at a lower starting dose (Badoux *et al.* 2011).

A further key feature of the clinical experience with lenalidomide is that it has efficacy in patients with "poor risk" disease. This was initially noted in a subgroup analysis of the first published trial in relapsed/refractory patients, which showed an ORR of 38% in patients with deletion of 17p or 11q (Sher *et al.* 2010). In an upfront study of lenalidomide in CLL, none of the established prognostic markers correlated with response, with patients who had adverse risk factors (including cytogenetics, CD38 and ZAP70) showing comparable responses to patients without these risk factors (Chen *et al.* 2010). However, there have been other conflicting reports about the efficacy of lenalidomide in patients with 17p deletion. This abnormality was associated with significantly impaired prognosis in two studies, with ORRs of 13% and 0% in relapse/refractory and treatment naïve patients respectively (Ferrajoli *et al.* 2008; Badoux *et al.* 2011). However, in a more recent study of lenalidomide in combination with rituximab conducted by the same group, the ORR for the 15 patients with 17p deletion was 53%, which was not significantly different from the ORR for patients without 17p deletion (70%; $P = 0.35$). Once again, other established prognostic

**Table 1.9 Summary of published clinical trials using lenalidomide in CLL**

| Relapsed/Refractory | | | | |
|---|---|---|---|---|
| **Reference** | **Patients** | **Treatment** | **Type** | **Response Rates** |
| (Chanan-Khan *et al.* 2006) | 45 patients; Relapsed/refractory Median of 3 prior $R_x$ Median age = 64 years | Lenalidomide 25mg, then 5 mg, with 5mg escalations to 25mg (21/28 days) | Phase II | ORR 58% (18% CR + 40% PR) (Data from 2007 update) |
| (Ferrajoli *et al.* 2008) | 44 patients; Relapsed/refractory Median of 5 prior $R_x$ Median age = 64 years | Lenalidomide 10mg, with 5mg escalations to 25mg (continuous) | Phase II | OOR 32 % (7% CR + 25% PR) 25% SD |
| (Wendtner *et al.* 2012) | 52 patients Relapsed/refractory Median of 4 prior $R_x$ Median age = 66 years | Lenalidomide 2.5mg, with escalations to 20mg (continuous) | Phase II/III | ORR 12% (0% CR + 12% PR) SD 58% |
| **Previously untreated** | | | | |
| **Reference** | **Patients** | **Treatment** | **Type** | **Response Rates** |
| (Chen *et al.* 2010) | 25 patients Untreated Median age = 60 years | Lenalidomide 2.5mg, 5mg, 10mg (21/28 days) | Phase II | ORR 72% 20% CR + 52% PR 40% SD (Data from ASH 2012 update) |
| (Badoux *et al.* 2011) | 60 patients Untreated Median age = 71 years | Lenalidomide 5mg, with 5mg escalations to 25mg (continuous) | Phase II | ORR 65% (15% CR + 50% PR) 40% SD |
| **Lenalidomide based combinations** | | | | |
| **Reference** | **Patients** | **Treatment** | **Type** | **Response Rates** |
| (Brown *et al.* 2010) | 9 patients Untreated Median age = 59 years | Fludarabine 25mg/$m^2$ for 3 – 5 days Rituximab 375mg/$m^2$ on day 1 Lenalidomide 2.5mg (21/28 days) | Phase I | ORR 56% (11% CR + 44% PR) 11% SD |
| (Badoux *et al.* 2013) | 59 patients Relapsed/refractory Median of 2 prior $R_x$ Median age = 62 years | Rituximab 375mg/$m^2$ on days 1, 8. 15, and 22 in cycle 1; subsequent cycles day 1 Lenalidomide 10mg (continuous) | Phase II | ORR 66% (12% CR + 54% PR) |
| (Shanafelt *et al.* 2013) | 44 patients (34 patients received lenalidomide) | Pentostatin 2mg/$m^2$ on day 1 Cyclophosphamide 600mg/$m^2$ on day 1 Rituximab 375mg/$m^2$ on day 1 (100mg/$m^2$ first dose in cycle 1) Lenalidomide consolidation (after completing PCR) 5mg, escalation up to 10mg | Phase II | Induction (PCR) ORR 93% (32% CR + 61% PR) Lenalidomide consolidation 24% improved depth of response (overall CR rate improved to 53% in lenalidomide patients) |

markers such as CD38 and *IGVH* mutation status did not correlate with ORR. Whether these observations translate into improved overall survival for poor risk patients remains to be determined.

Combinations of lenalidomide with other agents are now being tested in clinical trials. The addition of rituximab appears particularly attractive, given pre-clinical observations demonstrating enhanced NK cell responses after *in vitro* treatment with this agent (Wu *et al.* 2008). The ORR of the combination of lenalidomide and rituximab in relapsed/refractory patients was 66%, which compares very favourably to the ORRs of other salvage regimens such as FCR and BR. In contrast, the addition of fludarabine to lenalidomide and rituximab was not well tolerated, due to a combination of idiosyncratic drug reactions, tumour flare and myelosuppression (Brown *et al.* 2010). These problems made it difficult to deliver adequate amounts of fludarabine and/or lenalidomide to the patients, contributing to the poorer than expected ORR. Given that T-cell activation appears to be a crucial component of lenalidomide mechanism of action in CLL, it can be postulated that combinations with T-cell depleting agents such as fludarabine or alemtuzumab may be counterproductive. However the situation may be more complex, as there is some evidence that lenalidomide can still be effective after T-cell depletion by alemtuzumab and high-dose glucocorticoids (Arumainathan *et al.* 2011).

Following the example set in myeloma treatment, it is possible that lenalidomide may be useful as a consolidation or maintenance treatment. A recently published clinical trial examined the effect of induction therapy with pentostatin, cyclophosphamide and rituximab (PCR), followed by lenalidomide consolidation. In this context, lenalidomide consolidation improved the depth of response in 24% of eligible patients, with evidence suggesting that lenalidomide will extend time to retreatment (Shanafelt *et al.* 2013). Interestingly, correlative studies performed alongside this clinical trial contributed to by my group showed that anti-tumour T-cell immunological synapse activity was enhanced after PCR alone, and improved further with lenalidomide consolidation. Trials to investigate the combinations of lenalidomide with ofatumumab and dexamethasone, and as a maintenance treatment after FR/BR are ongoing (Ferrajoli *et al.* 2011; Chang *et al.* 2012; Chen *et al.* 2012; Ujjani *et al.* 2012).

Given the emergence and success of BCR-signalling inhibitors such as ibrutinib the future use of lenalidomide in CLL is unclear. It may well prove useful in combinations with these newer agents, or with more established chemotherapy drugs or monoclonal antibodies. As in myeloma, it may have a role to play as a consolidation or maintenance treatment, or as second line treatment in patients failing to respond. It may also be useful in enhancing T-cell and NK-cell responses post allogeneic stem cell transplantation or in combination with CAR T cells. Understanding the mechanism of action of lenalidomide in CLL will be key to developing rational novel drug combinations in the fight against this disease.

### *1.6.4* Lenalidomide in other haematological malignancies

Lenalidomide was first widely used in the treatment of multiple myeloma where it has been shown to have potent anti-tumour properties (Richardson *et al.* 2002). Lenalidomide has a dual mechanism of action in MM, involving direct tumouricidal activity that leads to MM cell death, and an immunomodulatory effect that keeps the tumour in remission and improves immune function (Davies and Baz 2010). The tumouricidal activity of lenalidomide is thought to be mediated by a combination of a direct cytotoxic effect, and by removal of bone marrow stromal cell (BMSC) support. BMSCs are thought to play an important role in the maintenance of myeloma cells *in vivo*, due to their ability to secrete factors such as IL6 and VEGF and to induce VEGF secretion by MM cells (Gupta *et al.* 2001). IL6 and VEGF are known to be important mediating MM cell growth and survival, and lenalidomide-induced downregulation of the production of these factors is thought to be an important part of the mechanism of action of this agent in MM (Lichtenstein *et al.* 1995; Breitkreutz *et al.* 2008) In addition, lenalidomide has a direct cytotoxic effect on MM cells, which is in contrast to CLL. This is thought to be a result of caspase activation, NFκB inhibition, and cell cycle arrest via upregulation of tumour suppressor genes including the cyclin-dependent kinase inhibitor p21 (Mitsiades *et al.* 2002; Davies and Baz 2010; Gandhi *et al.* 2010). However, as in CLL the immunomodulatory effect of lenalidomide is also important. Increased levels of IL2 in the MM microenvironment can potentiate T- and NK-cell proliferation, while an increase in their cytotoxic activity appears to be due to a combination of enhanced phagocytic function and antibody dependent cellular cytotoxicity (Hayashi *et al.* 2005). The activating effect of IMiDs on NK-cells appears to be particularly important in myeloma, where IMiDs have been shown to enhance NK-cell lytic activity against myeloma cell lines and autologous patient myeloma cells (Davies *et al.* 2001).

In addition, lenalidomide has also been found to have significant clinical activity in patients in MDS. Intriguingly, an early clinical study found that patients with 5q deleted MDS responded especially well to lenalidomide with an ORR of 83%, compared with ORRs of 57% for those with a normal karyotype and 12% for those with other karyotypic abnormalities (List *et al.* 2005). This observation sparked a great deal of interest in whether the genes that were normally encoded in the deleted part of chromosome 5q were important in the mechanism of action of lenalidomide. As a result of this several candidate genes were identified as being potentially important in

lenalidomide sensitivity, including *PP2A*, *ERG1*, *CDC25C*, and *CTNNA1* (Padron *et al.* 2011). Subsequent work showed that two dual specific phosphatases, Cdc25C and PP2Aα, which are co-regulators of the $G_2$-M cycle checkpoint, were inhibited by lenalidomide (Wei *et al.* 2009). The 5q deleted MDS progenitors were already haplodeficient for PP2A and Cdc25, making them especially sensitive to cell arrest induced by further inhibition of phosphatase activity by lenalidomide. Further investigation revealed that secondary resistance to lenalidomide in 5q deleted patients was associated with upregulation of *PP2Acα* with consequent restoration of p53 activation and hypo-proliferative anaemia (Wei *et al.* 2012). Similar reasoning provided the rationale for investigation of the tumour expression gene *SPARC*, which is also encoded on the region that is commonly affected in 5q deleted MDS patients (Pellagatti *et al.* 2007). Incubation with lenalidomide was found to up-regulate expression of this gene back to normal levels, potentially restoring its anti-proliferative, anti-adhesive, and anti-angiogenic functions.

## 1.7 Aims and objectives

This project has two principle aims. The first is to characterise the nature of the T-cell defect in CLL, focusing on the expansion of $CD8^+$ T cells in the peripheral blood of CLL patients. Given that an expansion of $CD8^+$ T cells in the peripheral blood is usually seen as part of the normal immune response to acute viral infections, a critical component of this will be to test the hypothesis that CLL peripheral blood T cells are phenotypically and functionally exhausted, analogous to observations in chronic viral infections. As part of this investigation there will be particular emphasis on assessing the differentiation state of CLL T cells, both in terms of cytokine secretion profiles and transcription factor expression.

The second aim is to investigate the potential of reversing these T-cell defects. The immunomodulatory drug lenalidomide is known to be clinically efficacious in CLL, an effect which relies on induction of an anti-tumour immune response rather than a direct cytotoxic effect. This project will investigate the mechanism of action of lenalidomide in CLL, with the hope that an increased understanding of this area will allow for further characterisation of the nature of the immune defect in this disease, and the development of novel immunotherapeutic approaches.

# Chapter 2: Materials and Methods

## 2.1 Ethical considerations

Ethical approval for a study entitled "The impact of the tissue microenvironment and immune system on haematological malignancies" was confirmed by the East London & The City Health Authority Local Research Ethics Committee 3. The research ethics committee (REC) reference number was 05/Q0605/140. This allowed for the use of stored samples of human biological material (e.g. blood, lymph nodes, bone marrow) and also covered the use of new samples of human biological material, from both patients with haematological malignancies, and healthy donors.

## 2.2 Patient samples

Samples of peripheral blood from patients with CLL were obtained from the tissue bank maintained by the Centre for Haemato-Oncology at Barts Cancer Institute. This tissue bank is maintained according to the Human Tissue Act 2004 (license no. 12199). All patients had signed consent forms to allow storage of specimens for research purposes after appropriate counselling in accordance with the Declaration of Helsinki. Further peripheral blood samples were also obtained from the tissue core maintained by the CLL Research Consortium (CRC), a United States-based multi-institutional programme project, funded by the National Institute of Health. The CRC tissue core is maintained according to the guidelines established by the Health Insurance Probability and Accountability Act (HIPAA). Similarly, all patients had been consented for sample storage in accordance with the Declaration of Helsinki, and any studies had to have been approved by the Institutional Review Board (IRB) of the relevant CRC site(s), of which Barts is the only non-USA based site.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated from buffy coats or buffy cones supplied by the National Blood Service, which were specifically designated for research use. Where additional clinical data was required (e.g. for purposes of age-matching) PBMCs were separated from peripheral blood samples kindly donated by known donors who were all were appropriately counselled and consented. Where required, the CMV serostatus of patients and healthy donors was determined by the Virology Department at the Royal London Hospital.

## 2.3 Cell thawing and freezing

Up to $10^8$ human cells were suspended in a solution of 90% foetal calf serum (FCS)(PAA Laboratories Ltd.) + 10% dimethylsulphoxide (DMSO)(Fisher Scientific). Up to 1.5ml cell suspension was then transferred to 2ml cryovials (Nunc), which were placed in freezing containers (Nalgene) at -80$^o$C overnight to allow gradual cooling at a rate of approximately 1$^o$C/minute. They were subsequently transferred to liquid nitrogen for long term storage.

On removal from liquid nitrogen the cells were rapidly thawed at 37$^o$C. The contents of the cryovial were then transferred to 10 – 20mls of full medium (RPMI 1640 + 10% FCS (both PAA Laboratories Ltd.) with 25mg gentamicin (Gibco)). The cell suspension was then rapidly washed to minimise toxicity from the DMSO.

## 2.4 Separation of PBMCs from whole blood/buffy coats/cones

Venous blood from patients with CLL was drawn into standard 6 – 10ml vacutainer tubes spray coated with ethylenediaminetetraacetic acid (EDTA) anticoagulant (BD). The anticoagulated blood was subsequently diluted in an equal volume of phosphate-buffered saline (PBS). Buffy cones from the National Blood Service were diluted 5-fold with 4 volumes of PBS due to the higher concentration of mononuclear cells. Up to 35ml of cell suspension was then layered on to 15ml Lymphoprep (Axis Shield) in 50ml Falcon tubes. These were then centrifuged at 1500rpm for 25 minutes at room temperature with the brake off. Lymphoprep has a specific gravity of 1.077 and thus mononuclear cells are found at the 1.077/plasma interface after centrifugation. This interface was carefully removed with a pastette and washed twice in full medium or MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin, and 2mM EDTA) (Miltenyi Biotec) prior to cell counting. Cell counting was performed on the Vi-cell XR, an automated cell counter (Beckman Coulter).

## 2.5 Cell Separation Protocols

### *2.5.1 Negative selection using magnetic microbeads*

Negative selection was performed by use of a magnetic cell sorting protocol developed by Miltenyi Biotec (**Figure 2.1**). MACS® LS columns, QuadroMACS® magnet, reagents and cell isolation kits were used as part of this protocol (Miltenyi Biotec). MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin, and 2mM EDTA) was prepared diluting MACS BSA Stock Solution 1:20 with autoMACS™ Rinsing Solution. The cells and buffer were kept cold (2-8$^{o}$C) throughout the isolation procedure to prevent capping of antibodies on the cell surface and non-specific cell labelling. PBMCs were isolated by density gradient centrifugation as described above. After washing and cell counting, the cells were resuspended in 40µl buffer per $10^7$ total cells. 10µl of biotin antibody cocktail per $10^7$ total cells was then added, and the cell suspension was then incubated for 10 minutes at 4$^{o}$C. Subsequently a further 30µl of buffer and 20µl of anti-biotin microbeads per $10^7$ total cells were added. The cell suspension was mixed and then incubated for an additional 15 minutes at 4$^{o}$C. The cells were then washed by adding 10-20x labelling volume of buffer and then resuspended at a concentration of up to 1 x $10^8$ cells in 500µl of buffer. The LS columns were prepared by rinsing with 3ml MACS buffer during the wash step. The cell suspension was applied to the column and washed through with 3 x 3ml of buffer. The entire effluent was collected, which was enriched for the unlabelled cells. Post separation the fractions were counted and aliquots removed for analysis of cell purity by flow cytometry. The remaining cells were used for downstream applications, or frozen as described above.

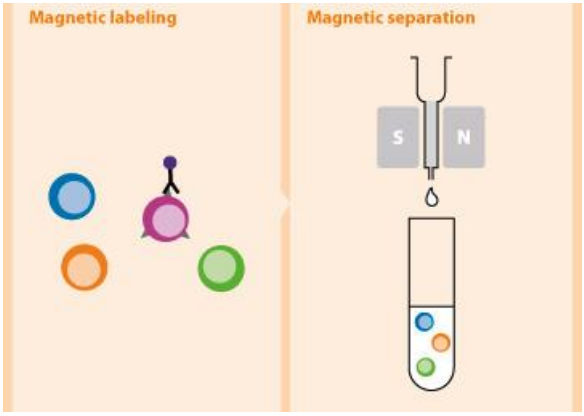



**Figure 2.1 Negative selection using immunomagnetic cell sorting**

https://www.miltenyibiotec.com/Products-and-Services/MACS-Cell-Separation/MACS-Technology/Separation-strategies.aspx

### *2.5.2 Positive selection using magnetic microbeads*

Positive selection was also performed by use of a magnetic cell sorting protocol developed by Miltenyi Biotec (Figure 2.2). MACS® LS columns, MACSbuffer, QuadroMACS® magnet, reagents and cell isolation kits were similarly used as part of this protocol (Miltenyi Biotec). PBMCs were isolated by density gradient centrifugation as described above. After washing and cell counting, the cells were resuspended in 80µl buffer per $10^7$ total cells and 20µl of antibody conjugated microbeads per $10^7$ total cells were added. The cell suspension was mixed and then incubated for 15 minutes at 4-8$^{o}$C. The cells were then washed by adding 10-20x labelling volume of buffer and subsequently resuspended at a concentration of up to 1 x $10^8$ cells in 500µl of buffer. The LS columns were prepared by rinsing with 3ml MACS buffer during the wash step. The cell suspension was applied to the column and washed through with 3 x 3ml of buffer. The LS column was removed from the magnetic field and flushed through with 5ml MACS buffer by firmly pushing the plunger into the column. This labelled cell fraction was counted and aliquots taken for analysis of cell purity by flow cytometry. The remaining cells were used for downstream applications such as bioassays or RNA extraction, or frozen as described above.

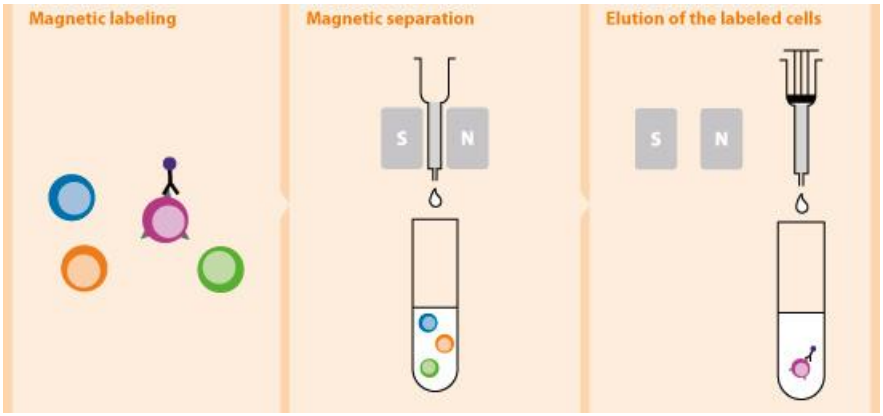



**Figure 2.2 Positive selection using immunomagnetic cell sorting**

https://www.miltenyibiotec.com/Products-and-Services/MACS-Cell-Separation/MACS-Technology/Separation-strategies.aspx

### *2.5.3 Immunomagnetic cell sorting using the AutoMACS™ Pro Separator*

Immunomagnetic cell sorting was also performed using the AutoMACS™ Pro Separator (Miltenyi Biotec). This automated approach has two main advantages. Firstly, the ability to use variable flow rates through the magnet and single/double column separations allowed for greater flexibility when selecting for different frequencies of target cells with different levels of target antigen expression. Secondly, the automated labelling and separation reduced the potential for human error, particularly when separating multiple samples. After density gradient centrifugation or cell culture, PBMCs were washed in autoMACS running buffer (Miltenyi Biotec) and counted. The cells were then resuspended in 80µl autoMACS running buffer per $10^7$ total cells for positive selection programs, and 40µl buffer per $10^7$ total cells for negative selection programs. The total volume of the cell suspensions were then programmed into the separator, along with the appropriate reagents for the target cell type, and the optimised cell separation strategy. The following cell separation programs were available (**Table 2.1**):

| **Positive selection programs:** | |
|---|---|
| POSSEL | Positive selection in standard mode: isolation of cells with normal antigen expression and frequencies higher than 5%; use if purity is the highest priority |
| POSSEL_S | Positive selection in sensitive mode: isolation of cells with low antigen expression and frequencies higher than 5%; use if yield is the highest priority |
| POSSELD | Positive selection in standard mode I, double column program: for isolation of rare cells in low elution volume |
| POSSELDS | Positive selection in sensitive mode, double column program: for isolation of rare cells with low antigen expression |
| POSSELD2 | Positive selection in standard mode II, double column program: for isolation of rare cells if purity is the highest priority |
| POSSELWB | Special positive selection in special mode, double column program: for isolation of cell subsets from whole blood |
| **Negative selection programs:** | |
| DEPLETE | Depletion in standard mode: for removal of cells with normal to high antigen expression and results in better target cell yield |
| DEPLETES | Depletion in sensitive mode I: removal of cells with low antigen expression and results in better target cell purity |
| DEPL05 | Depletion in sensitive mode II: removal of cells with low antigen expression and results in stringent depletion of cells |
| DEPL025 | Depletion in sensitive mode III: removal of cells with low antigen expression and results in stringent depletion of cells |

**Table 2.1 autoMACS® Pro Cell Separation programs**

### *2.5.4 Separation of T and NK cells from CLL PBMCs using the autoMACS Pro*

The low frequencies of T and NK cells in the peripheral blood of patients with CLL meant that selection of these subsets at a high purity was particularly challenging. The following strategy was used to isolate $CD3^+$, $CD4^+$ and $CD8^+$ T cells, and $CD56^+$ NK cells using the autoMACS Pro system (**Table 2.2**):

| **Step** | **Reagent** | **Cell separation Program** |
|---|---|---|
| Pre-depletion of CLL cells | | |
| If white blood count > 15 | CD19 microbeads | possel_s |
| If white blood count < 15 | CD19 microbeads | possel |
| Negative selection of: | | |
| $CD3^+$ T cells | Pan T cell Isolation Kit II | deplete |
| $CD4^+$ T cells | $CD4^+$ T cell Isolation Kit II | deplete |
| $CD8^+$ T cells | $CD8^+$ T cell Isolation Kit | depletes |
| NK cells | NK isolation kit | depletes |

**Table 2.2 Strategy for the separation of T and NK cells from CLL PBMCs**

This approach generally resulted in $CD4^+$ T cells with a purity of >95%, $CD8^+$ T cells with a purity of >90%, and NK cells with a purity of >85% by flow cytometry.

## 2.6 T-cell stimulation *in vitro*

For most assays, T cells were stimulated by using anti-CD3 antibodies, with or without co-stimulation using anti-CD28 antibodies. The HIT3a anti-CD3 antibody (BD) was optimised for use in solution at concentration of 1µg/ml with 5µg/ml soluble anti-CD28 (clone CD28.2, eBioscience) where required. This combination induced a strong proliferative response in healthy T cells. Where immobilized anti-CD3 antibodies were required for stimulation, 100µl – 1ml of a 10µg/ml solution of mouse anti-human CD3 (clone: OKT3 - eBioscience) was used to coat the bottom of flat bottom 6-, 12-, 24-, or 96-well plates (Corning). The plates were incubated at $4^{o}C$ overnight, washed twice with full medium, before the relevant cell suspension was added. T cells were also stimulated using magnetic beads coated with mouse anti-human anti-CD3 and anti-CD28 (Dynabeads® Human T cell expander; Invitrogen). These were prepared by washing three times in PBS with the aid of a magnet to separate the beads from the supernatant. The beads were resuspended at a concentration of $1 \times 10^{8}$ beads/ml and then added to the T-cell suspension at a starting ratio of 3:1 beads:T cells and mixed thoroughly.

For the analysis of intracellular cytokine production, phorbol 12-myristate 13-acetate (PMA) and ionomycin are widely used for T-cell stimulation. Cells were initially stimulated for 1 hour with 50 ng/ml PMA (Sigma Aldrich) and 1µg/ml ionomycin (Sigma Aldrich) and incubated at $37^{o}C$; 5% $CO_2$. 0.066% monensin (Golgistop BD) was then added and the cultures were then incubated for a further 4 hours (5 hours PMA and ionomycin stimulation in total). Monensin is a protein transport inhibitor, and has been demonstrated to significantly increase the ability to detect cytokine-producing cells by immunofluorescent staining (Jung *et al.* 1993).

A similar protocol was used for assessment of degranulation, although cells were stimulated with 1µg/ml staphylococcal enterotoxin B (SEB, Sigma) with the addition of 10µl/ml anti-CD107a-AlexaFluor647, instead of PMA and ionomycin. The fluorochrome was added during the incubation period as the CD107a protein gets internalised after translocation to the surface of the cell (Betts *et al.* 2003). As above, the cell cultures were incubated for 1 hour with SEB and anti-CD107a-AlexaFluor647, prior to addition of 0.066% monensin for 4 hours further incubation at $37^{o}C$; 5% $CO_2$.

## 2.7 Flow cytometry

This has been used for several purposes during this project including: purity of cell fractions after isolation procedures; lymphocyte phenotyping; intracellular cytokine expression; intranuclear transcription factor expression; assessment of lymphocyte proliferation and degranulation. A list of fluorochrome conjugated antibodies is given in the appendix. In general, a minimum of 10,000 - 20,000 events were used for each analysis and so 2 x $10^5$ cells were used at the start of these staining protocols. Where the cells of interest were a relatively rare subset (e.g. T cells in PBMCs from CLL patients), the starting number of cells was increased proportionally to account for this. The centrifugation steps were all performed at 1200 rpm for 5 minutes at 4$^o$C or room temperature, dependent on whether the cells were live or fixed respectively.

### *2.7.1 Surface staining*

PBMCs or pre-isolated lymphocytes were used after the thawing, cell separation, or culture procedures described above. All staining procedures on live cells were performed at 2 - 8$^o$C to avoid antibody capping and internalisation. The cells were transferred to 5ml polystyrene round-bottom tubes (BD), washed twice with 2ml PBS+2% FCS, prior to centrifugation at 1200 rpm for 5 minutes at 4$^o$C. They were then resuspended in 100µl PBS+2% FCS prior to addition of the fluorochrome conjugated antibody. Each antibody was optimised to determine the volume used for surface staining – this ranged from 1 – 20 µl. Where multiple colours were being analysed a master mix was prepared to ensure consistency of staining. The samples were then incubated for 30 minutes at 4$^o$C in the dark. The cells were then rewashed in 2ml PBS+2% FCS, centrifuged, and then resuspended in 400µl PBS+2% FCS. 4'6-diamidino-2-phenylindole (DAPI) was added to this final cell suspension at a concentration of 250ng/ml to allow for live/dead discrimination. The cells were kept on ice in the dark until flow cytometry was performed.

### *2.7.2 Intracytoplasmic staining (cytokines)*

DAPI cannot be used for live-dead discrimination with intracellular staining. DAPI stains dead cells because their cell membranes are more permeable, allowing the dye into the nucleus where it binds A-T rich regions of DNA. All intracellular staining protocols include a permeabilisation step, which will be mean that all cells will subsequently stain with DAPI. Therefore, live-dead discrimination was performed using a fixable viability dye (eBioscience) before performing surface staining. PBMCs or lymphocytes were washed twice with 2ml PBS and subsequently resuspended in 1ml PBS with fixable viability dye at a concentration of 1µl/ml. The cells were then washed in 2ml PBS+2% FCS, centrifuged, and then resuspended in 100µl PBS+2% FCS for surface staining as above. After surface staining the cells were washed with 2ml PBS+2% FCS, centrifuged and then resuspended in 200µl cold IC fixation buffer (eBioscience). The samples were then incubated at room temperature for 20 minutes in the dark. Without washing 2ml of 1x permeabilisation buffer (eBioscience) was added and the samples re-centrifuged. A further wash was performed in 1x permeabilisation buffer before the cells were centrifuged and resuspended in ~100µl 1x permeabilisation buffer for intracytoplasmic staining. A relevant volume (typically 1 - 2µl) of anti-cytokine antibody was then added at this stage and incubated for 30 minutes in the dark at room temperature. The samples were then washed in 1ml of 1x permeabilisation buffer, centrifuged, and resuspended in 400µl for PBS+2% FCS for flow cytometric analysis.

### *2.7.3 Intranuclear staining (transcription factors)*

Fixable viability dye and surface staining were performed in the same manner as for intracytoplasmic staining. After surface staining the cells were washed with 2ml PBS+2% FCS, centrifuged and then resuspended in 1ml of a formaldehyde based fixation/permeabilisation buffer (Fixation/Permeabilization Concentrate and Diluent, eBioscience). The samples were then incubated at room temperature for 30 - 60 minutes in the dark. Washing and permeabilisation was performed as above. The cells were then centrifuged and resuspended in 100µl 1x permeabilisation buffer for intranuclear staining. A relevant volume (typically 1 - 2µl) of anti-transcription factor antibody was then added at this stage and incubated for 30 minutes in the dark at room temperature. The samples were then washed in 2ml of the permeabilisation buffer, centrifuged, and resuspended in 400µl for PBS+2% FCS for flow cytometric analysis.

### *2.7.4 Carboxyfluorescein Succinimidyl Ester (CFSE) staining for proliferation*

For assessment of proliferation, cells were stained prior to cell culture with CFSE. Thawed cells were washed twice in pre-warmed (37$^{o}$C) serum free media (RPMI 1640 (PAA Laboratories Ltd.) + 25mg gentamicin (Gibco)) and counted. Cells were resuspended at a concentration of 2 x $10^{7}$/ml in serum free media. CFSE was diluted to make a 10μM working stock in pre-warmed serum free media. The cell suspension and CFSE working solution were mixed in equals volumes to achieve a final cell concentration of 1 x $10^{7}$/ml, and a final concentration of CFSE of 5μM. This resulting mixture was rapidly vortexed to ensure full distribution of CFSE, and incubated at 37$^{o}$C 5% $CO_2$ for 10 minutes. The cell suspension/CFSE staining reaction was then quenched by adding 10 – 20mls of cold (4$^{o}$C) full medium and then left to stand for five minutes. The cells were then washed three times in full medium prior to re-suspension for cell culture. Extra cells were stained purposes of CFSE compensation.

### *2.7.5 Typical multicolour flow panel design*

Combinations of fluorochromes were optimised to allow for performing multicolour flow cytometry. This had the dual advantages of enabling greater characterisation of cells with respect to both phenotype and function, and higher throughput and data gathering. Up to 8 colours were commonly used: some example "fluorochrome panels" as used on the Fortessa flow cytometer (BD) are shown (**Table 2.3**):

| **Laser** | Violet | Blue | Blue | Red | Red | Red | Yellow/ Green | Yellow/ Green |
|---|---|---|---|---|---|---|---|---|
| **Filter** | 450nm | 530nm | 695nm | 670nm | 730nm | 780nm | 585nm | 780nm |
| **Fluorochrome** | DAPI/ Pacific Blue | FITC | PerCP/ PerCP-Cy5.5 | APC/ AF647 | AF700 | APC-Cy7/ AF780 | PE | PE-Cy7 |
| **Surface Staining** | DAPI | PD1 | CD8 | CD160 | CD19 | CD4 | CD244 | CD3 |
| **Intracellular Staining** | CD3 | IFNγ | CD8 | CD45RA | CD19 | Fix-Via Dye | CCR7 | CD4 |

**Table 2.3 Examples of multicolour flow panels used**

Up to 8 colours were used for flow cytometry. DAPI was used as the live/dead discriminator for live cells, whereas fixable-viability dye eFluor780 (eBioscience) was used as the live/dead discriminator for permeabilised cells. For common used antigens (e.g. CD19, CD3, CD4, CD8) a wide range of directly conjugated antibodies were commercially available, whereas antibodies to less commonly used antigens (e.g. CD160, CD244) were often only available conjugated to FITC, PE, or APC. Therefore these colours were reserved for "target antigens".

### *2.7.6 Controls*

Unstained controls were used in all experiments to set the voltages on the flow cytometer. Fixation and permeabilisation of cells alters their forward and side scatter characteristics and so controls for these experiments were prepared in the same way as the sample tubes. Isotype controls were used for surface staining experiments where there was not expected to be a significant population of target antigen negative cells. Isotype controls were not used for intracellular staining, because most antibodies exhibit some non-specific binding which is a particular problem when intracellular epitopes and binding sites are available after permeabilisation. Therefore for anti-cytokine staining the anti-cytokine antibody was used its own control with the cells in the unstimulated state (Ormerod 2000). This was complemented by the use of internal controls within the experimental design. Flourescence minus one (FMO) controls were also used for certain experimental conditions, although as with isotype controls their reliability was reduced in the context of intracellular staining.

Compensation was performed using single stained controls when the cells were known to express the target antigen, or with compensation beads when the target antigen was under investigation. Anti-mouse immunoglobulin compensation beads (CompBeads, BD) were prepared by adding a drop each of negative control beads and anti-mouse compensation beads to a 5ml polystyrene round-bottom tube. A separate tube was prepared for each fluorochrome. A relevant volume of Igκ mouse anti-human flourochrome conjugated antibody was added to each tube, and incubated at 4$^{o}$C for 30 minutes in the dark. The beads were then washed with 2ml PBS+2% FCS and resuspended in 400µl for PBS+2% FCS for flow cytometric analysis. Cells were used to prepare a single stained sample for compensating the live-dead discrimination dye, and to provide an unstained control for settings voltages on the cytometer.

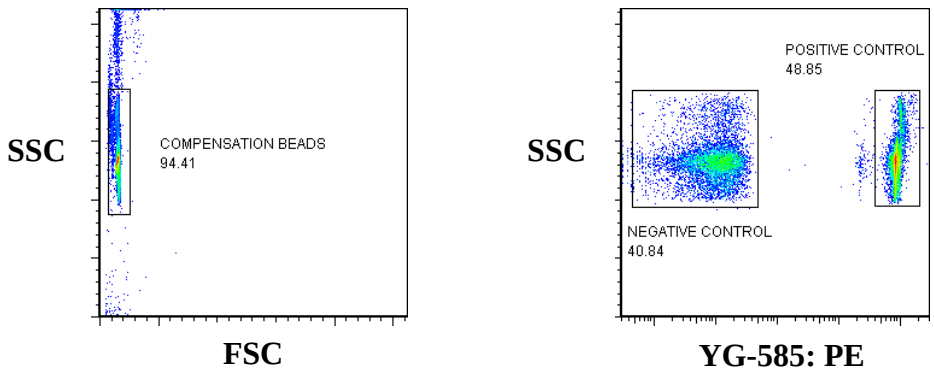



**Figure 2.3 An example of compensation beads for a single stained control (PE)**

### *2.7.7 Flow cytometric acquisition and analysis*

Initially a Cyan (Dako) and FacsCalibur (BD) and subsequently LSRFortessa (BD) were used for data acquisition. FlowJo (Tree Star Inc) software was used for analysis. The unstained control tube was primarily used to set voltages, although the single stained cell or compensation beads controls were also run prior to data recording. Analysis proceeded by defining the fluorochrome negative and positive populations on the single stained controls to allow for spectral compensation. For cells, these populations were defined on singlet-cells, and for beads, these were defined on the beads themselves, gating out any debris. These populations were then used to define a compensation matrix, which was subsequently applied to the test samples. After compensation, the following gating strategy was applied to the test samples. First, a gate was applied on the basis of DAPI/fixable viability dye and side scatter (SSC) to exclude dead cells from the analysis (A). A second gate was applied to the live cells to exclude doublets from the analysis, on the basis of SSC-area (SSC-A) and SSC-width (SSC-W)/pulse width (B). A third gate was then applied to the live-singlets to exclude debris from the analysis, on the basis of forward scatter (FSC) and SSC characteristics (C). For purity checks this would select all live-singlet-cells, for other analyses this gate could be adjusted to select a subpopulation (e.g. lymphocytes) based on FSC/SSC characteristics. Therefore all subsequent analysis of fluorochrome intensity was performed on compensated live-singlet-cells. A minimum of 10,000 events of the cells of interest were acquired when running samples on the cytometer.

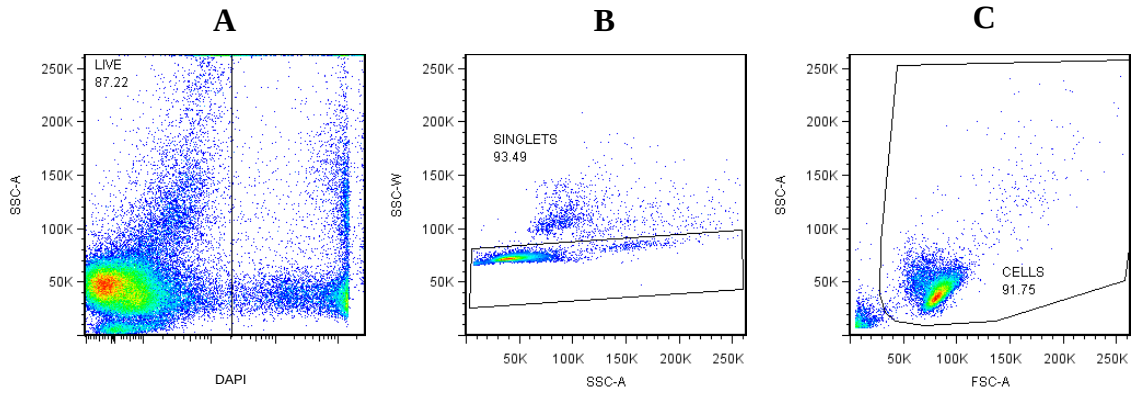



**Figure 2.4 Gating Strategy for Flow Cytometry**

(A) Events that have not stained for DAPI are gated on to exclude dead cells. (B) Single events (singlets) are gated on to exclude cell clumps (doublets/triplets etc). (C) Events with the forward and side scatter characteristics of cells are gated on to exclude debris and any contaminating red blood cells.

## 2.8 RNA extraction

RNA extraction was initially performed using the RNAeasy Mini Kit (Qiagen). However, there were consistent problems with genomic DNA contamination, so a switch was made to the RNAeasy Plus Mini Kit (Qiagen), which includes a step for the elimination of genomic DNA. The Qiagen kit contained all reagents apart from the QIAshredder (also Qiagen), 70% ethanol (VWR), and β-mercaptoethanol (Sigma Aldrich).

### *2.8.1 RNA extraction*

A total of 7 x $10^6$ isolated lymphocytes were used for RNA extraction. This provided satisfactory quantities of RNA without overloading the column and reducing purity. The cells were centrifuged for 1200 rpm x 5 minutes at room temperature to obtain a cell pellet. The supernatant was completed removed before the addition of 600µl Buffer RLT Plus with 1% β-mercaptoethanol. The lysate was pipetted directly into a QIAshredder spin column placed in a 2ml collection tube, and centrifuged for 2 minutes at 14,000 rpm. The homogenized lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 seconds at 14,000 rpm. 600µl of 70% ethanol was added to the homogenized lysate, which was thoroughly mixed by pipetting. 600µl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and pulsed for 15 seconds at 14,000 rpm. The flow-through was discarded and this step was repeated with any further sample. The RNeasy spin column was then washed with 700µl of Buffer RW1 and pulsed for 15 seconds at 14,000 rpm to wash the spin column membrane. The flow-through was discarded prior to the addition of 500 µl Buffer RPE to the column. The samples were then pulsed for another 15 seconds at 14,000 rpm to wash the spin column membrane, and the flow-through was discarded. A further 500 µl Buffer RPE was added to the RNeasy spin column, but this time the centrifugation was performed for 2 minutes at 14,000 rpm to dry the spin column membrane. The RNeasy spin column was placed in a fresh 2 ml collection tube and centrifuged for a further 60 seconds to ensure that no ethanol was carried over during RNA elution. The RNeasy spin column was then placed in a 1.5 ml collection tube, and 30µl of RNase-free water was added directly to the spin column membrane. A 4µl aliquot was taken for determination of RNA purity and integrity, and both the extracted RNA and test aliquot were rapidly frozen at -80$^o$C for long term storage.

### *2.8.2 RNA spectrometry to determine quantity and quality of RNA*

RNA spectrometry was performed to assess RNA quantity and quality on a Nanodrop spectrophotometer using 1.5µl of the RNA solution. The absorbance was measured at 230, 260, and 280 nm wavelengths. The ratio of absorbance at 260nm:280nm indicates purity and should be approximately 2.0 for good quality RNA. A reduction in the ratio indicates greater absorbance at 280nm which implies protein contamination. Similarly absorbance at 230nm indicates phenol contamination which is reflected in a fall in the ratio of absorbance at 260nm:230 nm to < 1.8. The RNA extracted using the Qiagen kits was usually of very high purity and no further precipitation steps were needed.

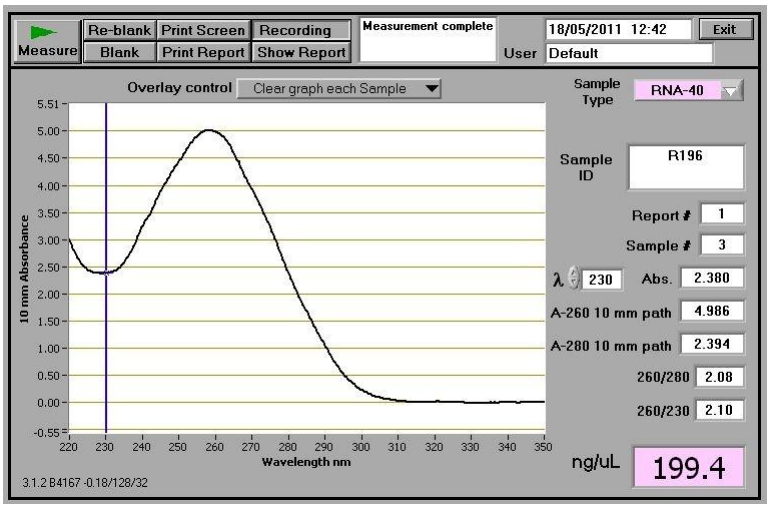



**Figure 2.5 RNA Spectrometry to determine quantity and quality of RNA**

Shows high quality RNA at a concentration of 199.4 ng/µl

### *2.8.3 RNA electrophoresis to determine integrity*

Due to the omnipresence of RNases, and the instability of RNA, a check of RNA integrity was performed on all RNA samples using the Agilent 2100 Bioanalyzer. This uses electrophoretic separation of nucleic acid fragments through an interconnected set of micro channels to produce information about the amount and integrity of total RNA. Gel-dye mix is loaded onto a chip using a priming device. Up to 12 samples of 1µl RNA solution at a concentration of 50 – 500ng/µl are then loaded with a standard RNA ladder. The samples are then driven through the gel-dye mix by electrophoresis, generating an electropherogram of fluorescence against time. Intact RNA should show to sharp peaks corresponding to the 28S and 18S subunits of ribosomal RNA. Agilent have developed a RNA integrity number (RIN) as a measure of integrity ranging from 1 representing most degraded, 10 most intact. A RIN above 6 is sufficient for most downstream applications, but virtually all samples had RINs greater than 8.

## 2.9 Gene expression microarrays (Affymetrix)

The protocol documented in the Affymetrix GeneChip® 3' IVT Express Kit User Manual was used to synthesis fragmented and purified aRNA for hybridisation to the microarrays. The quantity and purity of the starting RNA was assessed by spectrometry, and the integrity confirmed by RNA electrophoresis as above. The protocol recommended between 50 – 500ng starting RNA: due to cell numbers 250ng was used for both CLL and healthy B and $CD4^+$ T cells, and 125ng for both CLL and healthy $CD8^+$ T cells and NK cells. The quantity and integrity of RNA was retested after *in vitro* transcription and fragmentation (**Figure 2.6**)

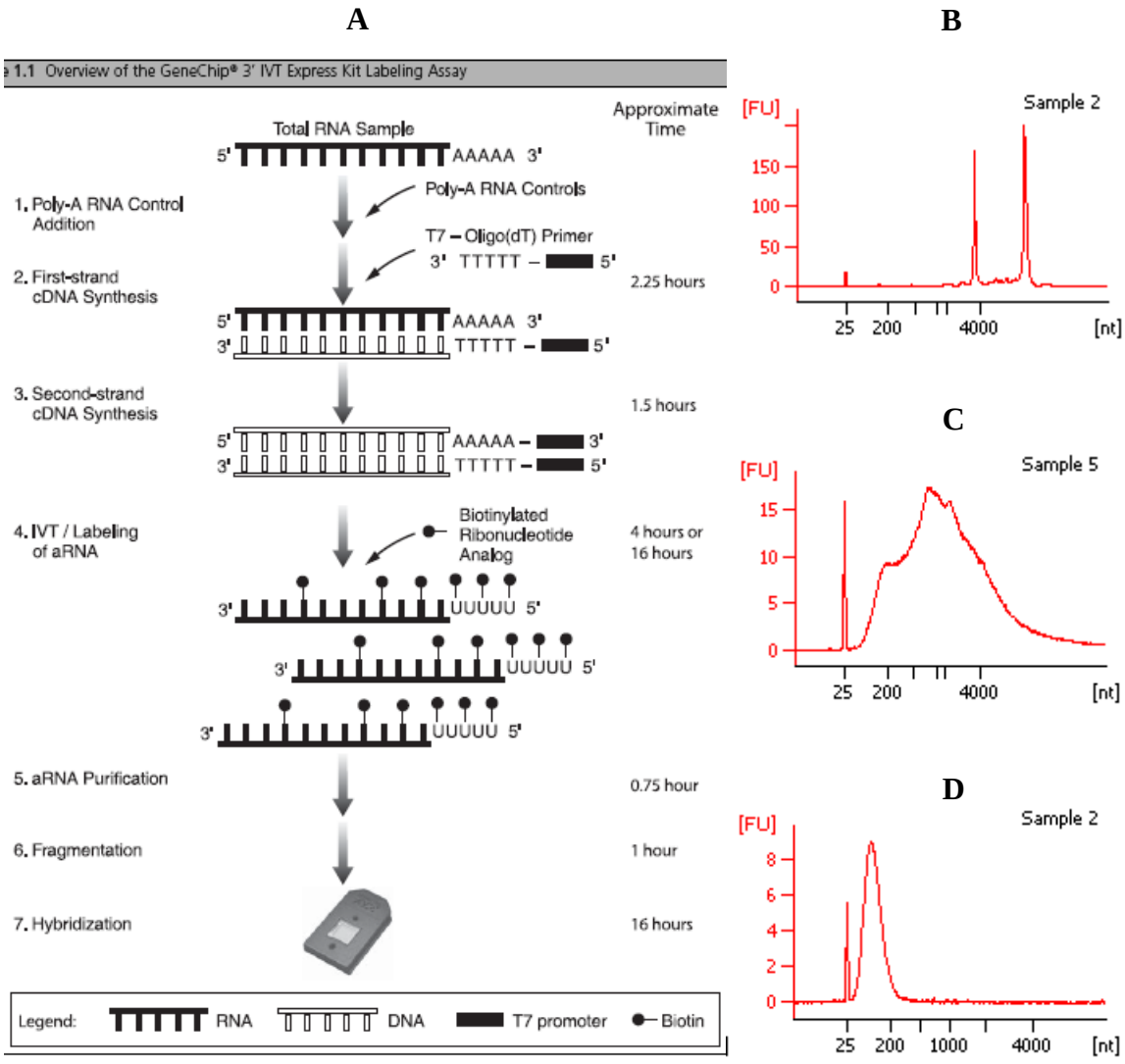



**Figure 2.6 Overview of the GeneChip® 3' IVT Express Kit Labelling Assay**

(A) Summary of the GeneChip® 3' IVT Express Kit Labelling Assay workflow. Electropherograms of intact starting RNA with clear ribosomal peaks (B), aRNA post *in vitro* transcription (C), and after fragmentation (D).

### *2.9.1 Reverse transcription to synthesise first-strand cDNA*

All reagents were included in the GeneChip® 3' IVT Express Kit. Samples were prepared on ice in 200µl PCR tubes. Reactions were assembled using "master mixes" to ensure consistency of reagent concentrations between samples.

Assembly of **First Strand Master Mix** (per sample):

| | |
|---|---|
| First-Strand Buffer Mix | 4µl |
| First-Strand Enzyme Mix | 1µl |
| **Total volume** | **5µl** |

5µl of the First-Strand Master Mix was then added to 5µl of Total RNA/poly-A control mixture which was prepared as follows:

| | |
|---|---|
| Total RNA sample | variable |
| Nuclease-free water | variable |
| Diluted Poly-A Controls* | 2µl |
| **Total volume** | **5µl** |

*A set of Poly-A controls were supplied with the GeneChip® 3' IVT Express Kit – these were designed to provide exogenous positive controls to monitor the labelling process. For 250ng of RNA, the control stock was diluted 1:200000 in four sequential dilutions (1:20; 1:50; 1:50; 1:4). For reactions using RNA extracted from low numbers of $CD8^+$ T cells and NK cells the Poly-A controls were omitted to allow for the use of a greater volume of target total RNA (5µl instead of 3 µl).

After mixing and brief centrifugation, the samples were incubated for 2 hours at 42$^o$C, before being placed on ice and immediately prepared for second strand cDNA synthesis.

### *2.9.2 Second Strand cDNA synthesis*

Assembly of **Second Strand Master Mix** (per sample):

| | |
|---|---|
| Second-Strand Buffer Mix | 5µl |
| Second-Strand Enzyme Mix | 2µl |
| Nuclease-free water | 13µl |
| **Total volume** | **20µl** |

20µl of the Second-Strand Master Mix was then transferred to each 10µl cDNA sample. After mixing and brief centrifugation, the samples were incubated for 1 hour at 16$^o$C, followed by 10 minutes at 65$^o$C, before being placed on ice and immediately prepared for *in vitro* transcription.

### *2.9.3 In Vitro Transcription (IVT) to synthesise labelled aRNA*

Assembly of **IVT Master Mix** (per sample):

| | |
|---|---|
| IVT Biotin Label | 4µl |
| IVT Labelling Buffer | 20µl |
| IVT Enzyme Mix | 6µl |
| **Total volume** | **30µl** |

30µl of the Second-Strand Master Mix was then transferred to each 30µl double stranded cDNA sample. After mixing and brief centrifugation, the samples were incubated for 16 hours at 40$^{o}$C, before being placed on ice and immediately prepared aRNA purification.

### *2.9.4 aRNA purification*

The aRNA elution solution was pre-heated to 55$^{o}$C for at least 10 minutes. While this was heating the **aRNA Binding Mix** was assembled (per sample):

| | |
|---|---|
| RNA Binding Beads | 10µl |
| aRNA Binding Buffer Concentrate | 50µl |
| **Total volume** | **60µl** |

60µl of aRNA Binding Mix was then added to each sample, and the resultant mixture transferred to a U-Bottomed 96-well plate. 120µl of 100% ethanol were then added to each sample to enable aRNA binding, and the plate was then shaken for 3 minutes at 400rpm on a MixMate Shaker (Eppendorf). The RNA bound beads were then captured by leaving to stand on a magnet for 5 minutes. The supernatant was then aspirated without disturbing the magnetic beads. After removal of the plate from the magnet, 100µl of aRNA Wash Solution was then added and then the plate was shaken for 1 minute at 800rpm on the MixMate shaker. The plate was then transferred to the magnet and the beads re-captured as previously. The supernatant was then aspirated without disturbing the magnetic beads, and the beads were then rewashed with a further 100µl of aRNA Wash Solution. After magnetic bead capture, the plate was dried by shaking for 2 minutes at 1200rpm on the MixMate shaker. The RNA was then eluted by adding 50µl of the pre-heated aRNA elution solution. The plate was then shaken for 3 minutes at 1200rpm on the MixMate shaker, and the aRNA binding beads were recaptured on the magnetic stand. The supernatant, containing the eluted RNA was then transferred to a nuclease-free PCR tube, quantified by spectroscopy and the quality check by electrophoresis before freezing at -20$^{o}$C.

### *2.9.5 Fragmentation of labelled aRNA*

Fragmentation of the aRNA target before hybridisation onto the GeneChip probe array is critical in obtaining assay sensitivity. Therefore 15µg of purified aRNA was prepared for fragmentation by assembling the **aRNA fragmentation mixture** (49/64 Format – per sample):

| | |
|---|---|
| aRNA (15µg) | variable |
| 5x Array Fragmentation Buffer | 8µl |
| Nuclease-free Water | variable |
| **Total volume** | **40µl** |

After mixing and brief centrifugation, the samples were incubated for 35 minutes at 94$^{o}$C, before being placed on ice and immediately prepared for hybridisation.

### *2.9.6 Target hybridisation for Affymetrix U133Plus2.0 Microarrays*

Reagents for this step were contained in the GeneChip® Hybridisation, Wash and Stain kit. The **hybridisation cocktail** was prepared (per sample - the 20x Hybridisation Controls were heated to 65$^{o}$C for 5 minutes to completely resuspend the aRNA before aliquoting) :

| | |
|---|---|
| Fragmented and labelled aRNA (12.5µg) | 33.3µl |
| Control Oligonucleotide B2 (3nM) | 4.2µl |
| 20x Hybridisation Controls (bioB, bioC, bioD, cre) | 12.5µl |
| 2x Hybridisation Mix | 125µl |
| DMSO | 25µl |
| Nuclease-free Water | 50µl |
| **Total volume** | **250µl** |

The hybridisation cocktail was then heated for 5 minutes at 99$^{o}$C. During this time the array (Affymetrix Human Genome U133Plus2.0) was wetted with 200µl of Pre-hybridisation mix and incubated for 10 minutes at 45$^{o}$C with rotation. The heated hybridisation cocktail was then transferred to a 45$^{o}$C heat block for 5 minutes and then centrifuged to collect any insoluble material from the hybrisation mixture. The pre-heated array was removed from the hybridisation oven and the pre-hybridisation mix was replaced by the clarified hybridisation cocktail. The probe array was then hybridised for 16 hours at 45$^{o}$C rotating at 60rpm. The hybridised arrays were then analysed using the GeneChip Fluidics station 450. The approach used to analyse the data will be discussed in chapter 4.

## 2.10 Quantitative real-time polymerase chain reaction (QRT-PCR)

### *2.10.1 High Capacity cDNA reverse transcription*

Total RNA was reverse transcribed to produce cDNA for QRT-PCR using the high capacity cDNA reverse transcription kit (Applied Biosystems). The workflow for this procedure is illustrated in Figure 2.7. The total RNA was thawed on ice and re-quantified by RNA spectroscopy. 500-2000ng RNA was then diluted in an appropriate volume of RNAase free water to make up the total volume to 10µl. A **Reverse Transcription Master Mix** was assembled (per sample):

| | |
|---|---|
| 10x RT Buffer | 2µl |
| 25x dNTP Mix (100 mM) | 0.8µl |
| 10x RT Random Primers | 2µl |
| Multiscribe Reverse Transcriptase | 1µl |
| Nuclease-free Water | 4.2µl |
| **Total Volume** | **10µl** |

10µl of the Reverse Transcription Master Mix was then transferred to each 10µl total RNA sample. After mixing and brief centrifugation, the samples were incubated for 10 minutes at 25$^{o}$C, 120 minutes at 37$^{o}$C, and then 5 minutes at 85$^{o}$C. The cDNA placed on ice and then either frozen at -20$^{o}$C or immediately used for gene expression assays.

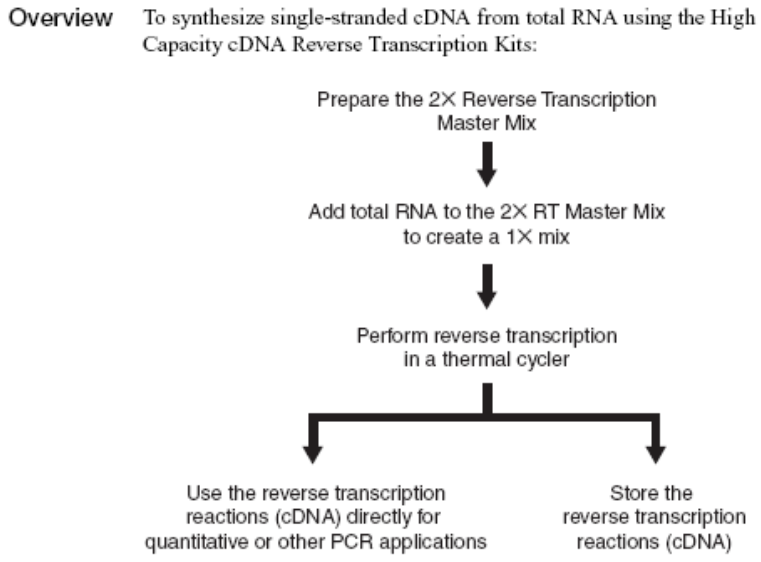



**Figure 2.7 Overview high capacity cDNA reverse transcription kit workflow**

### *2.10.2 Taqman® gene expression assays*

Applied Biosystems Taqman® Gene Expression assays were used to validate the changes in gene expression observed using the Affymetrix Genechip® arrays. Assays were chosen on the basis of whether they spanned an exon-exon junction of the associated genes and therefore would not detect genomic DNA (_m suffix: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/PCR/real-time-pcr/real-time-pcr-assays.html).

A **Taqman® Gene Expression Assay Master Mix** was assembled to ensure consistency between samples (per sample):

| | |
|---|---|
| 2x Taqman® Universal PCR Master Mix (Applied Biosystems) | 10µl |
| 20x Taqman® Gene Expression Assay | 1µl |
| RNA-ase Free Water | 7-8 µl |
| **Total Volume** | **18-19µl** |

The total volume of 18-19µl was then added to each well of a MicroAmp Fast Optical 96-well plate (Applied Biosystems) in a dedicated hood that was cleaned and UV decontaminated between uses. 1-2µl of cDNA template produced by the reverse transcription reaction was then added to make the final volume up to 20µl. Typically 50ng of cDNA in 1µl (50ng/µl) was used. Samples were loaded in duplicate with GAPDH (Applied Biosystems) as an endogenous control. An example of a plate design used is shown in **Table 2.4**.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| R127 CLDN12 | R127 CLDN12 | R127 F11R | R127 F11R | R127 CTNNA | R127 CTNNA | R127 TJP2 | R127 TJP2 | R127 RND1 | R127 RND1 | R127 GAPDH | R127 GAPDH |
| R131 CLDN12 | R131 CLDN12 | R131 F11R | R131 F11R | R131 CTNNA | R131 CTNNA | R131 TJP2 | R131 TJP2 | R131 RND1 | R131 RND1 | R131 GAPDH | R131 GAPDH |
| R139 CLDN12 | R139 CLDN12 | R139 F11R | R139 F11R | R139 CTNNA | R139 CTNNA | R139 TJP2 | R139 TJP2 | R139 RND1 | R139 RND1 | R139 GAPDH | R139 GAPDH |
| R144 CLDN12 | R144 CLDN12 | R144 F11R | R144 F11R | R144 CTNNA | R144 CTNNA | R144 TJP2 | R144 TJP2 | R144 RND1 | R144 RND1 | R144 GAPDH | R144 GAPDH |
| R150 CLDN12 | R150 CLDN12 | R150 F11R | R150 F11R | R150 CTNNA | R150 CTNNA | R150 TJP2 | R150 TJP2 | R150 RND1 | R150 RND1 | R150 GAPDH | R150 GAPDH |
| R155 CLDN12 | R155 CLDN12 | R155 F11R | R155 F11R | R155 CTNNA | R155 CTNNA | R155 TJP2 | R155 TJP2 | R155 RND1 | R155 RND1 | R155 GAPDH | R155 GAPDH |
| R174 CLDN12 | R174 CLDN12 | R174 F11R | R174 F11R | R174 CTNNA | R174 CTNNA | R174 TJP2 | R174 TJP2 | R174 RND1 | R174 RND1 | R174 GAPDH | R174 GAPDH |
| R179 CLDN12 | R179 CLDN12 | R179 F11R | R179 F11R | R179 CTNNA | R179 CTNNA | R179 TJP2 | R179 TJP2 | R179 RND1 | R179 RND1 | R179 GAPDH | R179 GAPDH |

**Table 2.4 Example of a QRT-PCR 96-well plate design with samples in duplicate**

Plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) and pulsed to 1500rpm. Reactions were performed on an Applied Biosystems 7900HT Fast Real Time PCR System using the standard thermal cycler protocol (50$^{o}$C for 2 minutes, 95$^{o}$C for 10 minutes, then 50 cycles of 95$^{o}$C for 15 seconds and 60$^{o}$C for 1 minute).

### *2.10.3 Principle of QRT-PCR: 5' nuclease assay*

QRT-PCR enables quantification of amplified DNA in real time. The Taqman® gene expression assays use sequence-specific oligonucleotide DNA probes labelled with a fluorescent reporter linked to the 5' end of the probe, (typically FAM™: 6-carboxyfluorescein) along with a minor groove binder (MGB) and non-fluorescent quencher (NFQ) at the 3'end of the probe. This system permits detection only after hybridisation of the probe with its complementary DNA target, resulting in significantly increased specificity. An exponential increase in the product targeted by the reporter probe at each PCR cycle causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter (**Figure 2.8**).

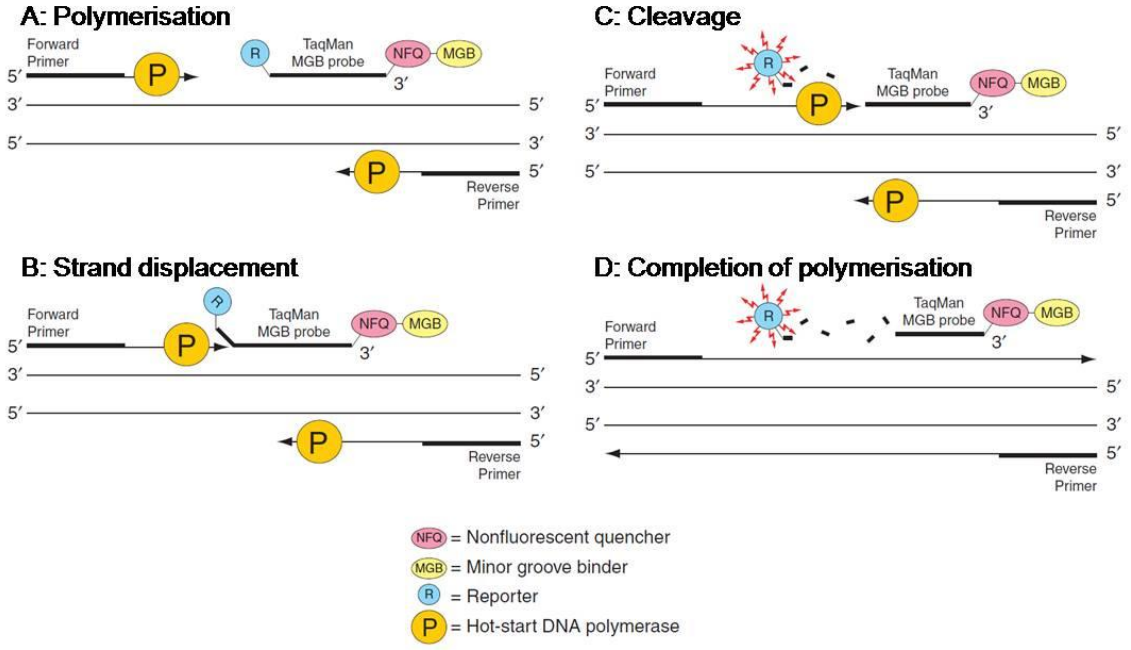



**Figure 2.8 Taqman® Chemistry: Principle of the 5' nuclease assay**

(A) During PCR, the Taqman MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. (A) and (B) When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Förster type energy transfer. (C) The DNA polymerase cleaves only probes that are hybridised to the target. Cleavage separates the reporter dye from the quencher dye resulting in increased fluorescence by the reporter. (D) Polymerisation of the strand continues, but because the 3' end of the probe is blocked, no extension of the probe occurs during PCR. Adapted from Taqman® Gene Expression Assays Protocol (Applied Biosystems).

### *2.10.4 QRT-PCR analysis*

Initial analysis was performed using the SDS2.3 and RQ Manager1.2 software for the 7900HT Fast Real Time PCR System (Applied Biosystems). Using this software a cycle threshold value ($C_T$) was automatically calculated for each sample. This value is the fractional cycle number at which fluorescence from that sample exceeds background levels. As fluorescence reflects the relative amount of DNA present in the reaction, the $C_T$ value is reached more quickly (i.e. in fewer cycles) when there is a greater amount of target DNA in the sample. As each sample was run in duplicate, the average of the duplicate was taken where the standard deviation was <0.25. Where this was exceeded, the data was visualised. Where there was a clear outlier, this was excluded, and the $C_T$ value calculated from the remaining value. When it was unclear as to the true value, the sample was excluded for downstream analysis. The expression of each target was then normalised to GAPDH expression to give a $\Delta C_T$ value. This was calculated by subtracting the mean $C_T$ value for reference gene (GAPDH) from the mean $C_T$ value of the target gene. Where there were paired samples (e.g. with/without drug treatment), the $\Delta C_T$ value for the control sample was subtracted from the $\Delta C_T$ value for the treated sample to give a $\Delta\Delta C_T$ value. As the fluorescence doubles with each cycle during the exponential phase of the reaction, log base 2 of the $-\Delta\Delta C_T$ value ($2^{-\Delta\Delta CT}$) gives a measure of the absolute differences in cDNA between reactions. Where gene expression between unpaired samples was being compared (e.g. healthy versus CLL), the expression of the target gene relative to GAPDH ($2^{-\Delta CT}$) was used.

## 2.11 Western blotting

### *2.11.1 Extraction of total cell lysate*

For each protein sample at least 1 x $10^6$ of cells (5 x $10^6$ primary cells) was required to harvest enough total protein lysate for western blotting analysis. For western blotting analysis where the target protein had low expression, or for enzyme activity assays, significantly more primary cells (ideally 20-50 x $10^6$ primary lymphocytes) were required to give good results. Isolated or thawed cells were washed twice in cold (4$^o$C) serum-free buffer. PBS was used to prepare cells for lysing for western blotting. However a phosphate-free buffer such as Tris-buffered saline (TBS) was used to prepare cells for lysing for phosphatase assays. The cell suspensions (in 1ml of buffer) were then transferred to a pre-cooled 1.5ml Eppendorf, and centrifuged in a pre-cooled centrifuge for 5 mins at 2000rpm at 4$^o$C. During the centrifugation step the lysis buffer mixture was assembled:

| | Western Blotting | PP2A assay |
|---|---|---|
| CelLytic Cell Lysis Reagent (Sigma) | 980µl | 970µl |
| Protease Inhibitor Cocktail (Sigma) | 10µl | 10µl |
| Phosphatase Inhibitor Cocktail Set 2 (Sigma) | 10µl | 0µl |
| PMSF (Phenylmethylsulfonyl fluoride; Sigma) | 0µl | 10µl |
| DTT (Dithiothreitol; Sigma) | 0µl | 10µl |
| **Total Volume** | **1000µl** | **1000µl** |

After centrifugation, the supernatant was removed and 30 – 120 µl lysis buffer mixture added (volume dependent on size of cell pellet). The cell pellet was resuspended by pipetting and vortexed for 10 seconds. The lysis reaction was then incubated on ice for 15 minutes, with repeated vortexing every 5 minutes. After incubation, the extracts were re-vortexed before centrifuging for 10 minutes at 14000rpm at 4$^o$C. The supernatant containing the total protein extract was then transferred into fresh pre-cooled 1.5ml Eppendorfs and immediately analysed for protein concentration or frozen at -80$^o$C.

### *2.11.2 Determination of protein concentration (Bradford Assay)*

The Bradford Assay uses the dye Coomassie® (Brilliant Blue) G-250 to determine protein concentration. Coomassie® G-250 (Bio-Rad Protein Assay Dye Reagent Concentrate; BioRad) binds to protein forming a protein-dye complex which induces a change in the colour of the dye from reddish to blue, shifting the absorbance maximum of the dye from 465nm to 595nm. The absorption increase at 595nm is proportional to the protein concentration and is measured using a spectrophotometer (Bradford 1976). This assay uses defined BSA concentrations to produce a standard curve from which unknown protein concentrations can be calculated by linear regression. 100µl of 1× Bio-Rad reagent working solution was added to the wells of a flat bottomed 96-well plate using a multichannel pipette. A 0.5mg/ml BSA working solution was prepared and increasing volumes were added to the Bio-Rad reagent to produce a standard curve. 1µl of a 1:10 dilution of the sample protein lysates was then added to the Bio-Rad reagent in the test wells and mixed thoroughly. The protein concentration was then determined at 595nm using an Opsy MR 96-well plate reader

Example 96-well plate (triplicates):

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | S1 | S1 | X | X | X | X | X | X | X | X | X |
| S2 | S2 | S2 | X | X | X | X | X | X | X | X | X |
| S3 | S3 | S3 | X | X | X | X | X | X | X | X | X |
| S4 | S4 | S4 | X | X | X | X | X | X | X | X | X |
| S5 | S5 | S5 | X | X | X | X | X | X | X | X | X |
| T1 | T1 | T1 | X | X | X | X | X | X | X | X | X |
| T2 | T2 | T2 | X | X | X | X | X | X | X | X | X |
| B1 | B1 | B1 | X | X | X | X | X | X | X | X | X |

Standard1 = 100µl Biorad + 0µl BSA
Standard2 = 100µl Biorad + 0.5µl BSA
Standard3 = 100µl Biorad + 1µl BSA
Standard4 = 100µl Biorad + 2µl BSA
Standard5 = 100µl Biorad + 4µl BSA

Test1 = 100µl Biorad + 1µl Sample
Test2 = 100µl Biorad + 1µl Sample
Blank1 = 100µl Biorad
X = no sample

### *2.11.3 Western blotting*

Western blotting is a widely accepted technique used to detect specific proteins in cell lysates. It uses gel electrophoresis to separate denatured proteins by molecular weight. The proteins are then transferred to a polyvinylidene fluoride (PVDF) membrane, where they are stained with antibodies specific to the target protein. The following solutions were prepared:

**10x Tris Buffered Saline (TBS):**

| | |
|---|---|
| Distilled Water | 1 litre |
| TRIS-HCl (Sigma) | 24.2g |
| Sodium Chloride (Sigma) | 80g |
| **Adjust pH to 7.6** | |

**1x Tris Buffered Saline-Tween (TBS-T)**

| | |
|---|---|
| Distilled Water | 900ml |
| 10x TBS | 100ml |
| Tween20 (Sigma) | 1ml |

**Running buffer:**

| | |
|---|---|
| NUPAGE SOS running buffer (Invitrogen) | 50ml |
| (MOPS for proteins > 40KDa, MES for proteins < 40KDa) | |
| Distilled Water | 950ml |

**5% Milk Blocking buffer**

| | |
|---|---|
| TBS-T | 20ml |
| Dried Milk Powder (Marvel) | 1g |

**5% BSA Blocking buffer**

| | |
|---|---|
| TBS-T | 20ml |
| BSA (Sigma) | 1g |

The volume of cell lysate required for a constant amount of total protein (10-100µg) per sample was calculated and lysis buffer mixture was added to produce a fixed concentration/volume of protein. The samples (with equivalent amounts of total protein) were mixed with LDS sample buffer in a 3:1 (Sample:LDS Sample buffer) ratio. The samples were then denatured at 70$^{o}$C for 10 minutes in a hot block. During this period the gel chamber was prepared for electrophoresis. A Nupage 4-12% Bis-Tris 10-well gel was used for most experiments. The white tape was stripped off from the lower end and top insert removed without disrupting wells. The gel was placed in the gel chamber, which was then filled with MOPS/MES running buffer. To visualize the molecular weight of the proteins, a standard containing 9 recombinant proteins ranging in size from 20-220 kDa was prepared (MagicMark; Invitrogen). 3µl Magic mark was added to 22µl loading buffer, with 10µl of the resultant solution being added to the first gel pocket. Up to 25µl of the denatured protein samples were added to the remaining gel pockets. The lid of the gel cell was replaced, the electrodes were aligned and leads attached to the power unit. 200V of current was then applied for 40-50 minutes to allow the samples to run to the bottom of the gel. After the electrophoresis the gel was removed from the chamber and prepared for blotting. The iBlot device (Invitrogen) was used for this (**Figure 2.9**). The cassette was opened and the gel was placed on top of the anode, removing any excess parts of the gel. Pre-soaked filter paper was placed directly on top of the gel and any air bubbles removed. The cathode stack and disposable sponge were then applied and blotting was performed using programme P3 for 7 minutes. After


**Figure 2.9 Blotting using the iBlot Device**

the programme had run the membrane was removed and immediately transferred to the blocking solution. The subsequent protocol was optimised for each antibody used. Typically, the membrane was then blocked for 1 hour in 5% milk blocking buffer at room temperature on a rocker. The membrane was then incubated overnight at 4$^{o}$C on a rocker with an appropriate dilution of primary antibody (e.g. 1:1000). The membrane was then washed twice for 10 minutes in TBS-T. It was subsequently incubated with and appropriate dilution of secondary antibody (e.g. 1:2000 for Polyclonal goat anti-rabbit; Dako) for 1 hour at room temperature on a rocker. The membrane was then washed three times in TBS-T for 10 minutes before preparation for chemiluminescence.

The membrane was then read on a Fuji-film image reader LAS-4000 machine which had been pre-cooled to -30$^{o}$C. Following washing the membrane was soaked in 1ml of Novex® ECL Chemiluminescence Substrate Reagent Kit (Invitrogen). This was prepared by mixing reagent A (luminol) and reagent B (an enhancer) in equal volumes. The membrane was then incubated for 2-5 minutes in the dark at room temperature, before being transferred to the image reader. The images were then acquired: "super binning" was used first to determine the quality of the membrane and to check position; the final images were acquired using the "high resolution" setting. After image acquisition the membrane was washed three times with TBS-T and could be re-probed or stored as required. For short term (< 1 week) storage the membrane was stored in TBS-T at 4$^{o}$C, for longer term storage the membrane was dried between two sheets of Whatman paper, wrapped in cling film and stored at -20$^{o}$C.

## 2.12 Phosphatase assay

The activity of protein phosphatase 2A (PP2A) was determined using the PP2A Immunoprecipitation Phosphatase Assay Kit (Millipore). The kit was used according to the manufacturer's instructions with a few modifications. Cell lysates were prepared as described above.

### *2.12.1 Binding of anti-PP2A to beads*

An anti-PP2A monoclonal antibody (clone 1D6) was bound to beads as follows. A **Bead Binding Mixture** was assembled in a 1.5ml Eppendorf (per sample):

| | |
|---|---|
| pNPP Ser/Thr Assay Buffer | 300µl |
| Protein A agarose slurry | 40µl |
| anti-PP2A C (200µg/200µl solution) | 4µl |
| **Total volume** | **344µl** |

The resultant mixture was then incubated for 1 hour on a rotor at 4$^{o}$C. During this time the sample lysates were thawed and their protein concentration determined using the Bradford assay as above.

### *2.12.2 Enzyme assay*

After bead binding 100µg of total protein was added to the Bead Binding Mixture. The total volume was made up to 500µl using pNPP Ser/Thr Assay Buffer. The resultant solution was then incubated for 2 hours on rotor at 4$^{o}$C. After incubating the beads were washed 3 times with 700µl TBS, followed by one wash with 500µl Ser/Thr Assay buffer. A 1mM solution of Threonine Phosphopeptide was prepared by adding 1.1ml distilled water to the 1mg peptide supplied. 60µl of this diluted phosphopeptide was then added to each sample along with 20µl Ser/Thr Assay buffer. The reaction was then incubated for 10 minutes at 30$^{o}$C in a waterbath with continuous agitation before being placed back on ice. The Malachite Green Phosphate Detection Solution was prepared by adding 10µl of Solution B to each 1ml of Solution A provided in the kit. 100µl of this solution was then added to the wells of flat-bottomed 96-well plate. A phosphate standard was prepared by diluting 125µl of phosphate standard solution C with 1125µl of distilled water to make a 0.1mM working solution. This was used to prepare a phosphate standard curve as shown in **Table 2.5**. 25µl of the standards and samples were added to each of the wells (samples prepared in triplicate) which were then read at 630nm using an Opsy MR 96-well plate reader to determine phosphate concentration.

| | | | | | | |
|---|---|---|---|---|---|---|
| Volume of diluted stock (0.1mM) | 200µl | 160µl | 120µl | 80µl | 40µl | 0µl |
| Volume of distilled water | 50µl | 90µl | 130µl | 170µl | 210µl | 250µl |
| Picomoles of phosphate/ 25µl | 2000 | 1600 | 1200 | 800 | 400 | 0 |

**Table 2.5 Preparation of phosphate standard curve for phosphatase assay**

## 2.13 Cell conjugation assays and confocal microscopy

Confocal microscopy was used to assess the cellular localisation of target proteins, and to evaluate immunological synapse formation.

### *2.13.1 Cell conjugation assays*

T and B cells were isolated as previously described. Where thawed cells were used, these were "rested" in full medium for 1-2 hours or overnight as required. Healthy B cells or CLL cells ($1.5 \times 10^6$ cells) were washed in serum-free medium and then stained with 7-amino-4-chloromethylcoumarin CellTracker Blue for 30 minutes at 37°C. They were subsequently incubated in full medium for a further 30 minutes at 37°C, with or without 2μg/ml of staphylococcal superantigen cocktail (Sigma). After washing the cells were then centrifuged with an equal number of T cells at 1500rpm for 5 minutes to allow a cell pellet to form. The resultant cell conjugates were then incubated at 37°C for 10-15 minutes, gently resuspended, and then transferred onto and Poly-l-lysine coated glass slides using a cytofuge (Statspin).

### *2.13.2 Staining for confocal microscopy*

After transfer to the slides the cells were fixed in 3% methanol-free formaldehyde in PBS (TAAB Laboratories Equipment Ltd) for 15 minutes. The cells were then washed 3 times in PBS, and then permeabilised in 0.3% tritonx100 (Sigma) in PBS for 5 minutes. The cells were then washed a further 3 times in PBS, before being blocked with 0.1% BSA (Miltenyi Biotec)/PBS blocking solution for 10 minutes. If primary antibodies were being used, the cells were incubated with the relevant antibody diluted in goat serum buffer (Sigma) for 45 minutes at $4^oC$. The cells were then rewashed 3 times in PBS. A secondary staining solution was prepared with a 1:40 dilution of rhodamine phalloidin (Invitrogen) in goat serum buffer and appropriate secondary antibody if required (e.g. Alexa 488 anti-mouse IgG 1:500 dilution; Invitrogen). The cells were then incubated for a further 20 minutes at $4^oC$ in the dark. The cytofuge unit was then disassembled and the slides mounted with coverslips with fluorescent mounting medium (DAKO). Confocal microscopy was performed using a Zeiss LSM510 confocal laser-scanning microscope using a 63x objective. Images were acquired from the slides in a consistent manner: a minimum of 10 images /50 cell conjugates were acquired per condition.

## 2.14 Mass spectrometry

As several pharmacological agents used during the course of this project, mass spectrometric analysis was frequently performed to confirm drug stability and quantity. The stock solution of the agent under investigation was diluted as required (usually 1 in 1000) in 30% acetonitrile solution containing 0.1% formic acid in LC-MS grade water (all from Fisher Scientific). The diluted solution was infused into the mass spectrometer directly using 500µl Hamilton Syringe (Sigma-Aldrich, UK) at flow rate of 5µl/min into the triple stage quadrupole (TSQ) Vantage mass spectrometry system (Thermo Scientific, UK) equipped with a heated electrospray ion source. All experiments were carried out in positive ionization mode. Spray voltage was set at 3500 volts; sheath gas (nitrogen) flow rate was 20 arbitrary units. Argon was used as collision gas with pressure of 1.5 mTorr. The predicted molecular weight of the compound under investigation was used to find candidate molecules. These were then fragmented to establish a breakdown curve: the mass transitions could be used to confirm the identity of the parent compound. The ion count of the parent compound or principle fragment was compared with a freshly reconstituted standard for assessing drug stability. Data were acquired using Thermo Xcalibur software (version 2.1).

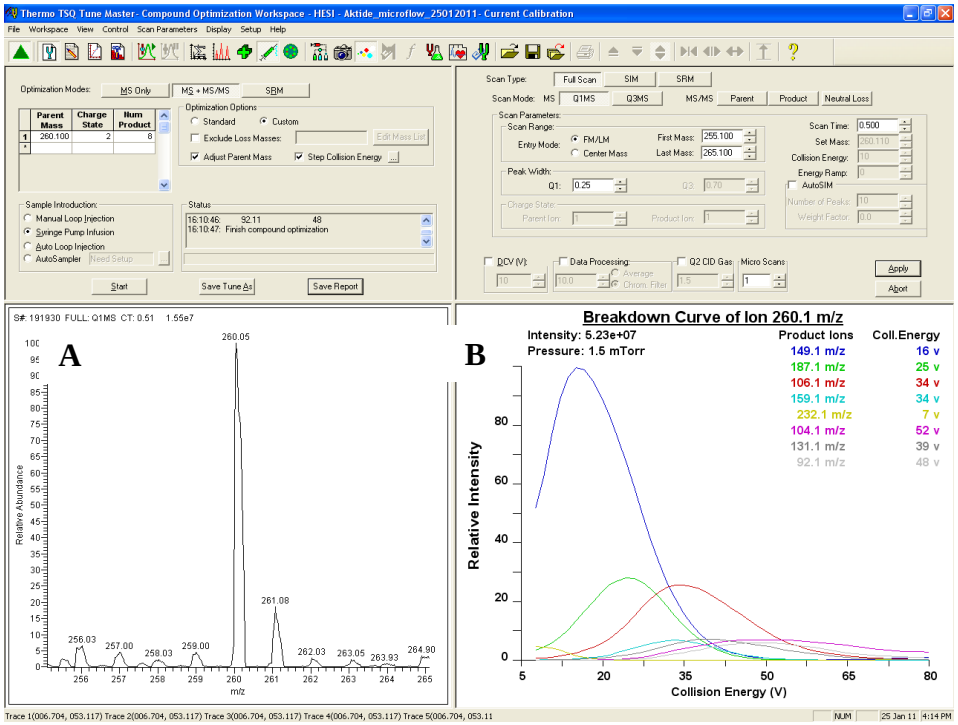



**Figure 2.10 Mass spectrogram of 10µM lenalidomide in DMSO**

(A) Mass spectrogram showing the presence of a parent compound with a mass of 260 with a high ion count of 1.5 x $10^7$. (B) When fragmented, this compound had a principle fragment with a mass of 149.1 consistent with the principle transition of lenalidomide (Liu *et al.* 2008).

# Chapter 3: Characterisation of T cells from CLL patients

## 3.1 Introduction

CLL is associated with profound defects in T-cell function, resulting in failure of anti-tumour immunity and increased susceptibility to infections. Despite this, it has been known for some time that there is actually an *increase* in absolute numbers of T cells in the peripheral blood in CLL, which is primarily accounted for by an increase in $CD8^+$ T cells, resulting in a fall in the CD4:CD8 ratio (Catovsky *et al.* 1974; Lauria *et al.* 1980; Herrmann *et al.* 1982; Mills *et al.* 1982; Platsoucas *et al.* 1982). A number of studies have linked these changes to prognosis, with early work demonstrating that there was greater expansion of $CD8^+$ T cells with advancing Rai stage (Herrmann *et al.* 1982), with a more recent study showing that inverted CD4:CD8 ratios predict shorter time to first treatment and reduced progression-free survival (Nunes *et al.* 2012). Another important feature of these T cells is that they show evidence of chronic activation, with upregulation of markers of activation such as CD69, HLA-DR and CD57, and down-regulation of CD28 and CD62L (Velardi *et al.* 1985; Rossi *et al.* 1996; Van den Hove *et al.* 1998; Van den Hove *et al.* 1998). This expansion of activated T cells has also been observed in the Eμ-Tcl1 mouse model of CLL, where a skewing of T-cell subsets towards antigen-experienced memory T cells, and development of clonal T-cell populations have been reported (Hofbauer *et al.* 2011).

An expansion of activated $CD8^+$ T cells in the peripheral blood is seen as part of the normal immune response to acute viral infections. During a primary immune response to viruses, naïve $CD8^+$ T cells expressing TCRs specific for viral peptides clonally expand and differentiate into effector $CD8^+$ T cells that control the primary infection. These effector T cells are then able to destroy any virally infected cells through the targeted secretion of perforin and granzyme from lytic vesicles. After the primary infection is cleared there is a contraction phase when most of these effector $CD8^+$ T cells die by apoptosis. However an effective immune response also produces a stable population of long lived memory $CD8^+$ T cells that can respond rapidly to clear secondary infections. These memory $CD8^+$ T cells are maintained in the absence of antigen by interactions with homeostatic cytokines such as IL7 and IL15, that mediate survival and self-renewal signals (Cui and Kaech 2010).

However some viruses, such as hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) in humans, and lymphocytic choriomeningitis virus (LCMV) in mice, have evolved mechanisms enabling them to evade effector immune responses. This leads to failure of immune responses to clear these viral infections, and persistence of viral antigens beyond the effector phase. This continued presence of antigen drives ongoing replication of effector T cells, leading to a number of phenotypic and functional changes that have been termed T-cell "exhaustion". In particular, there is a hierarchical loss of effector $CD8^+$ T-cell function, resulting in loss of proliferative capacity, impaired cytotoxicity, and reduced cytokine production. This is associated with increased expression of inhibitory receptors such as PD1, CD160 and CD244, and increased expression of transcription factors implicated in promoting exhaustion such as BLIMP1 and eomesodermin (Wherry 2011). Notably, gene expression profiling of exhausted $CD8^+$ T cells reveals a distinct transcriptional state with down-regulation of cytoskeletal genes leading to impaired immunological synapse formation and vesicle trafficking (Thoulouze *et al.* 2006; Wherry *et al.* 2007). We noted similarities in these changes to the alterations in gene expression that my group has previously observed in $CD8^+$ T cells in CLL patients (Gorgun *et al.* 2005).

One of the concepts in CLL that has emerged over the last few years is that chronic antigenic drive plays a key role in the pathogenesis of this disease. Observations such as the presence of stereotyped B-cell receptors, the importance of prognostic markers associated with BCR-signalling, and the success of therapies targeting BCR-signalling have highlighted the importance of chronic antigenic stimulation in driving the disease (Hamblin *et al.* 1999; Rosen *et al.* 2010). Therefore it would be reasonable to speculate that this continued antigenic drive could also result in persistent T-cell stimulation and activation. In light of this, it was hypothesized that the presence of an expanded population of activated $CD8^+$ T cells in the peripheral blood of CLL patients is a consequence of chronic antigenic stimulation of the T-cells compartment. Furthermore, it was also hypothesized that the functional defects represent a state analogous to the T-cell exhaustion that has observed in the context of chronic viral infections. Importantly, T-cell exhaustion has recently been reported in haematological malignancies, including adult T-cell leukaemia/lymphoma, chronic myeloid leukeamia, and acute myeloid leukaemia (Kozako *et al.* 2009; Mumprecht *et al.* 2009; Zhou *et al.* 2011).

However, a major potential confounding factor is previous cytomegalovirus (CMV) infection. CMV is known to have a profound influence on distribution of lymphocyte subsets in healthy individuals, with CMV seropositivity associated with driving the T-cell repertoire towards greater clonality in the elderly (Khan *et al.* 2002; Chidrawar *et al.* 2009). Subsequent reports have demonstrated an expansion of CMV-specific CD4+ and CD8+ T cells in patients with CLL, that have an effector phenotype (Mackus *et al.* 2003; Pourgheysari *et al.* 2010; Walton *et al.* 2010). It is therefore possible that the expansion of activated CD8+ T cells in the peripheral blood of CLL patients merely represents an immune response to CMV. As CMV is a chronic viral infection, it was crucial to control for this factor when testing the hypothesis that CLL T cells are "exhausted".

## 3.2 Aims and objectives

The primary aim of this work was to characterise T cells from patients with CLL and test the hypothesis that they are phenotypically and functionally "exhausted". The secondary aim was to assess the impact of CMV-serostatus on the function of T cells from CLL patients.

## 3.3 Materials and Methods

### *3.3.1 Patients and Samples*

Peripheral blood samples were obtained from 39 CLL patients from the tissue bank maintained by the Department of Haemato-Oncology of St. Bartholomew's Hospital, London, United Kingdom. Ethical approval was confirmed by the East London & The City Health Authority Local Research Ethics Committee, and written informed consent was obtained in accordance with the Declaration of Helsinki. All of the patients were untreated at time of blood withdrawal, and had a median age of 59 years (range 43 – 86). The patients had predominantly early stage CLL with 31/39 (79.5%) classed as having Binet stage A disease. Peripheral blood samples were also obtained from a control group of 20 healthy volunteers, who were age-matched with a median age of 61 years (range 49–72). The CMV serostatus of patients and healthy donors was determined by the Virology Department at the Royal London Hospital. 22/39 (56%) of patients and 13/20 (65%) of healthy donors were found to be CMV+.

### *3.3.2 Monoclonal antibodies*

The following directly conjugated monoclonal antibodies were used in this study: CD3-Pacific Blue, CD3-PECy7, CD4-PECy7, CD4-eFluor780, CD8-PerCPCy5.5, CD107a-AlexaFluor647, CD127-FITC, CD160-AlexaFluor647, CD197-PE, CD197-APC, CD244-PE, CD244-APC, TBET-PE, IFNγ-FITC, CTLA4-PE, TIM3-APC were all obtained from eBioscience. CD19-AlexaFluor700, CD45RA-FITC, CD122-PE, PD1-FITC, PD1-APC, IL2-PE, IL4-PE, TNFα-FITC were all obtained from BD Biosciences; Blimp1-PE was obtained from Santa-Cruz Biotec; LAG3-APC was obtained from R&D Systems. For confocal microscopy, unconjugated primary antibodies specific for CD107a and Granzyme B were from Abcam, and Alexa Fluor 488 and 647-labeled goat anti-mouse IgG were from Life Technologies.

### *3.3.3 Isolation of PBMCs and lymphocyte subsets*

Peripheral blood samples were diluted 1:1 with PBS prior to separation of PBMCs by density gradient centrifugation as described in section 2.4. $CD8^+$ T cells were negatively selected using the $CD8^+$ T cell Isolation Kit (Miltenyi Biotec) using the AutoMACS Pro as described in section 2.5.3.

### *3.3.4 Immunofluorescence staining and flow cytometric analysis*

Surface and intracellular staining for flow cytometric analysis was performed as described in section 2.7. Flow cytometry was performed on a BD Fortessa flow cytometer with subsequent analysis using FlowJo software (Tree Star). Analysis was performed on a minimum of 10,000 T-cell events after gating on live singlet cells.

### *3.3.5 T-cell stimulation*

For assessment of cytokine production, cells were stimulated with 50ng/ml phorbol myristate acetate (PMA) and 1µM ionomycin (Sigma). For assessment of degranulation, cells were stimulated with 1µg/ml staphylococcal enterotoxin B (SEB)(Sigma) with the addition of 10µl/ml anti-CD107a-AlexaFluor647.(Betts *et al.* 2003) For both experiments the cell cultures were incubated for 1 hour prior to addition of 0.66 µl/ml Golgistop (BD), before 4 hours further incubation at 37$^{o}$C; 5% $CO_2$. For assessment of proliferation, 1x10$^{7}$cells/ml were washed and stained with 5µM carboxyfluorescein succinimidyl ester (CFSE, eBioscience) for 10 minutes at 37$^{o}$C. After quenching and further washing, the cells were stimulated with 1µg/ml soluble anti-CD3 (clone HIT3a, BD) and 5µg/ml soluble anti-CD28 (clone CD28.2, eBioscience) for 72 hours.

### *3.3.6 Cytotoxicity assay*

Cytotoxicity assays were performed with Dr Alan Ramsay and Mrs Rewas Fatah. HLA-A*0201–expressing CD8$^{+}$ cells were stimulated in vitro with dendritic cells pulsed with a CLL $IgV_H$-derived peptide TLYLQMNSL weekly for 4 weeks, and killing of peptide-pulsed H2 cells was assessed. Target cells were labelled with 100µCi $^{51}$Cr (3.7MBq; NEN) and seeded in 96-well U-bottom microtiter plates at a concentration of 2.5 × 10$^{3}$/ml in triplicates. Effector cells were added in a ratio of 1:3, 1:10, and 1:30 and co-cultured for 4 hours at 37°C; 5% $CO_2$. After 4 hours the supernatants were harvested and the released $^{51}$Cr was measured in a γ-Counter (Wallac). Spontaneous release was determined by incubation of treated target cells in medium alone and maximum release was determined by resuspending the wells with 2% Triton X-100. Specific lysis was determined as previously described (Ramsay *et al.* 2008).

### *3.3.7 Cell conjugation assays and confocal microscopy*

Cell conjugation assays and confocal microscopy were performed with Dr Alan Ramsay and Mrs Rewas Fatah. Healthy B cells or CLL cells ($1 \times 10^6$ cells) were stained with 7-amino-4-chloromethylcoumarin CellTracker Blue and pulsed with 2μg/ml of staphylococcal superantigen cocktail (Sigma) for 30 minutes at 37°C. B cells were centrifuged with an equal number of $CD8^+$ T cells and incubated at 37°C for 10 minutes, plated onto poly-L-lysine–coated slides, and fixed for 15 minutes at room temperature with 3% formaldehyde. After fixation, the cells were permeabilised in 0.3% Triton (Sigma) for 5 min and blocked with 0.1% Bovine serum albumin (Miltenyi Biotec) in PBS for 10 minutes. Primary and secondary antibodies were applied sequentially for 30 minutes at 4°C in 5% goat serum (Sigma). Filamentous actin (F-actin) was stained with rhodamine phalloidin (Invitrogen). Confocal microscopy was performed using a Zeiss LSM510 confocal laser-scanning microscope using a 63x objective.

### *3.3.8 Statistical analysis*

All data sets were subject to normality testing using the Shapiro-Wilk normality test. Where all data sets could be accurately modelled by a Gaussian distribution an unpaired t test was used for analysis of differences between groups; where this was not the case the 2-sided Mann Witney *U* test was used. For comparison of 4 groups the Kruskal-Wallis test was used with Dunn's post test for multiple comparisons. *P* values of less than 0.05 were considered statistically significant.

## 3.4 Results

### *3.4.1 Increased numbers of CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells in the peripheral blood of patients with CLL*

Initial immunophenotyping showed an increase in absolute numbers of CD3$^+$CD8$^+$ (and CD3$^+$CD4$^+$ T cells circulating in the peripheral blood of patients with CLL compared to age-matched healthy controls. The expansion of CD3$^+$CD8$^+$ T cells ($P$ = 0.0004) was greater than the expansion of CD3$^+$CD4+ T cells ($P$ = 0.0036), resulting a fall in the CD4:CD8 ratio consistent with previous reports ($P$ = 0.021)(Catovsky *et al.* 1974; Lauria *et al.* 1980; Herrmann *et al.* 1982; Mills *et al.* 1982; Platsoucas *et al.* 1982)(**Figure 3.1)**.

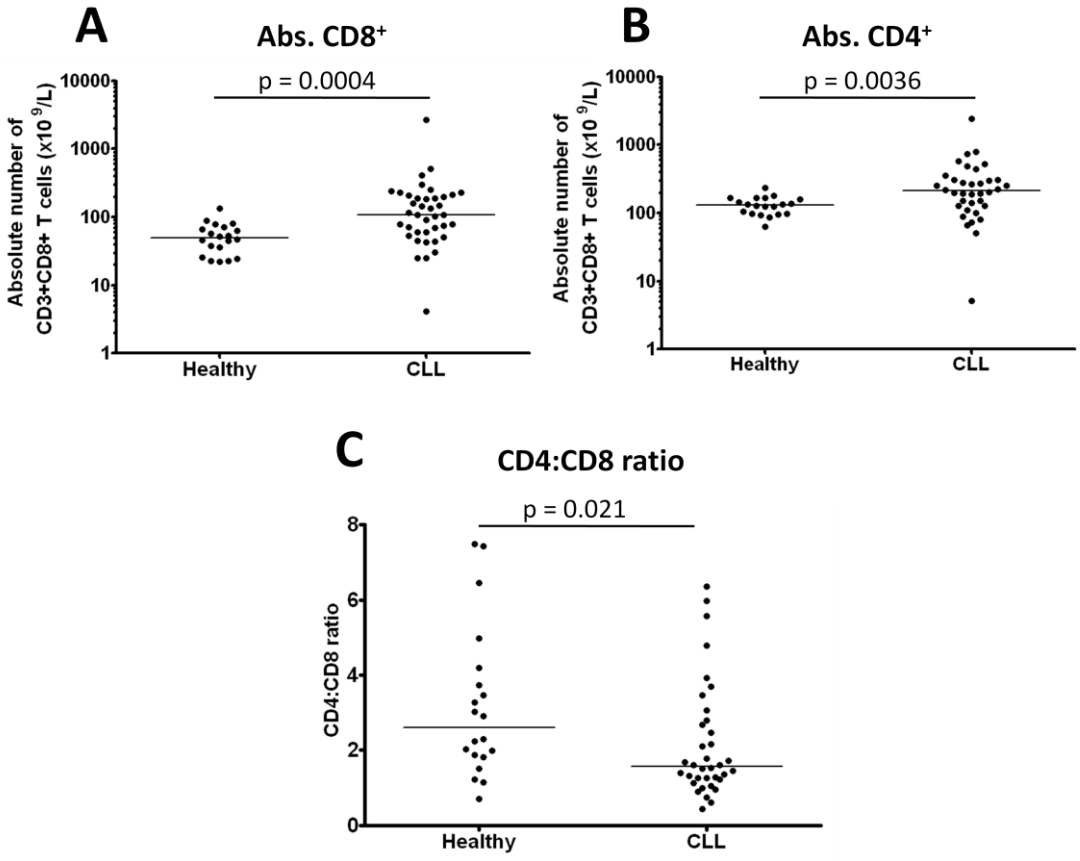



**Figure 3.1 Increased numbers of CD8$^+$ and CD4$^+$ T cells in the peripheral blood of patients with CLL**

Absolute numbers of CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells were measured in the peripheral blood of patients with CLL. CLL patients had increased absolute numbers of both CD3$^+$CD8$^+$ T cells (A) and CD3$^+$CD4$^+$ T cells (B) compared to health controls. (C) The expansion of CD3$^+$CD8$^+$ T cells was greater, resulting in a fall in the CD4:CD8 ratio.

### *3.4.2 Increased expression of PD1, CD160 and CD244 on T cells from CLL patients*

Increased expression of CD160, CD244 and PD1 are key features of T-cell exhaustion (Wherry 2011). These molecules can act as negative regulators of lymphocyte activation when bound by their respective ligands, herpes virus entry mediator (HVEM), CD48, and programmed death ligand-1 (PDL1)(Freeman *et al.* 2000; Cai *et al.* 2008; Blackburn *et al.* 2009). Immunophenotyping of peripheral blood $CD3^+$ T cells from patients with CLL was compared with healthy controls, to determine whether they expressed these exhaustion markers. Compared with controls, there was a significant increase in both the percentage and absolute numbers of both $CD4^+$ and $CD8^+$T cells from CLL patients expressing CD244, CD160 and PD1 (**Figures 3.2 and 3.3**).

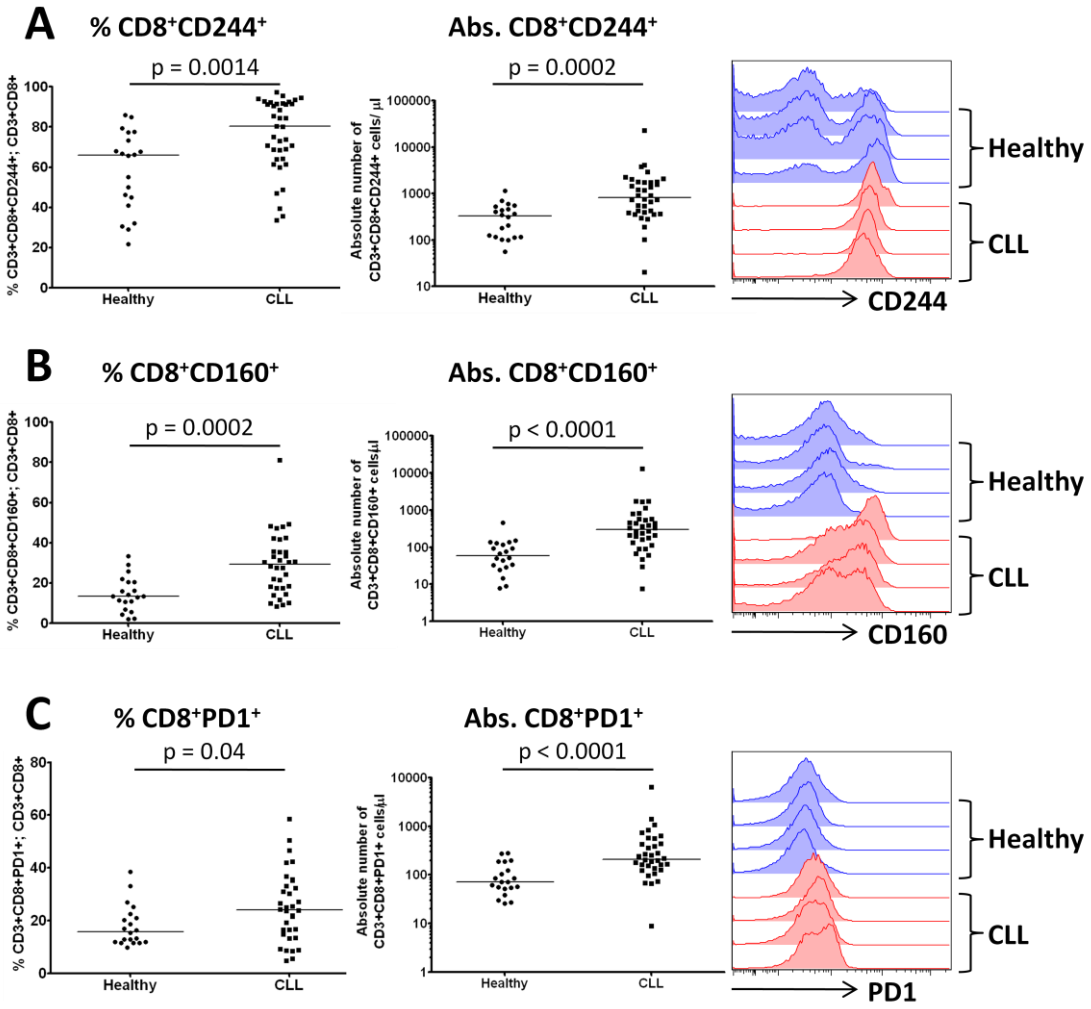



**Figure 3.2 Increased expression of PD1, CD160 and CD244 on $CD3^+CD8^+$ T cells from patients with CLL**

The expression of (A) CD244, (B) CD160 and (C) PD1 were assessed on $CD8^+$ T cells from patients with CLL and healthy age-matched controls. Significant increases in both the proportion, and the absolute number, of T cells expressing these molecules were observed.

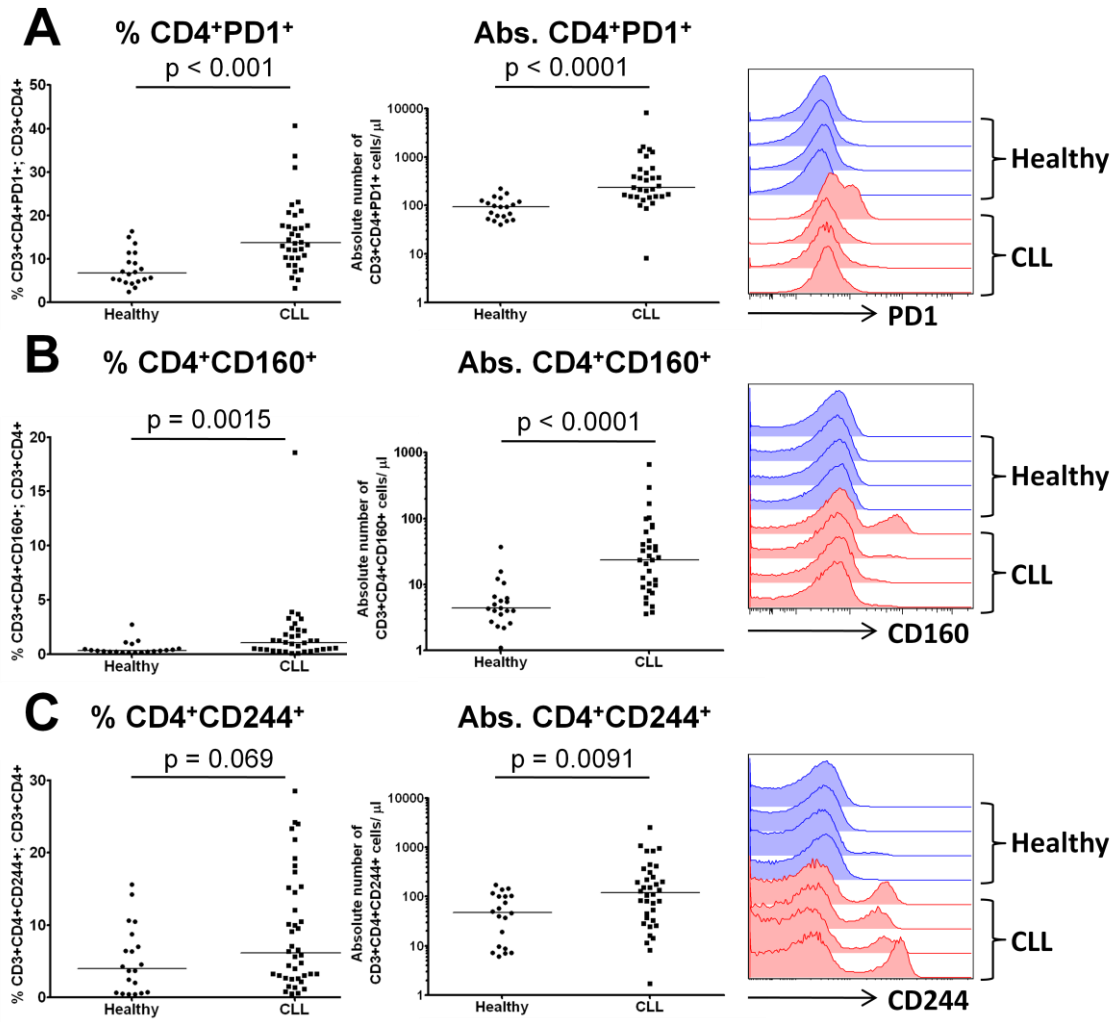



**Figure 3.3 Increased expression of PD1, CD160 and CD244 on $CD3^+CD4^+$ T cells from patients with CLL**

The expression PD1, CD160, and CD244 on $CD4^+$ T cells from patients with CLL and healthy age-matched controls was measured by flow cytometry . There was a significant increase in numbers of PD1+ (A) and CD160+ (B) and CD244+ (C) $CD3^+CD4^+$ T cells in CLL patients compared to healthy controls.

### *3.4.3 Expansion of PD1$^{+}$BLIMP1$^{HI}$ T cells in the peripheral blood of CLL patients*

B-lymphocyte induced maturation protein 1 (BLIMP1) has been shown to be important in driving CD8$^{+}$ T cell exhaustion in chronic viral infections (Shin *et al.* 2009). Expression of PD1 identified a subset of T cells with high expression of BLIMP1 (PD1$^{+}$BLIMP1$^{HI}$), that were expanded in both the CD3$^{+}$CD8$^{+}$ ($P < 0.0001$) and CD3$^{+}$CD4$^{+}$ ($P < 0.0001$) compartments in CLL (**Figure 3.4**). In contrast, expression of CD160 and CD244 did not identify subsets of T cells with upregulation of BLIMP1.

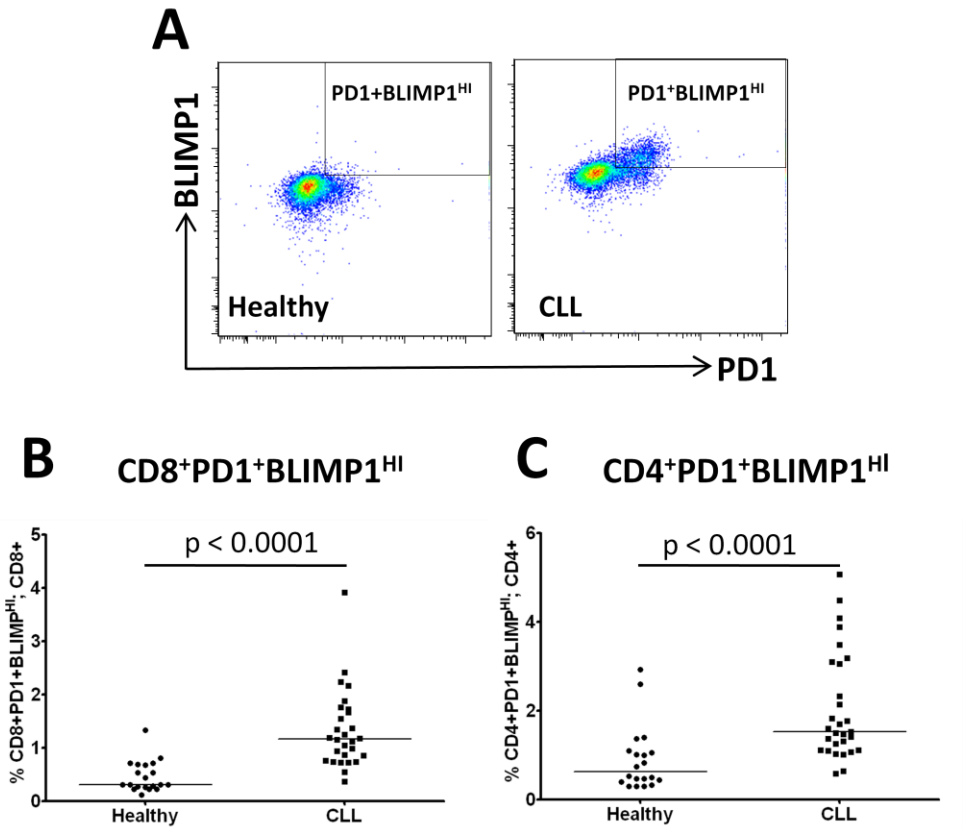



**Figure 3.4 Expansion of PD1$^{+}$BLIMP1$^{HI}$ T cells in the peripheral blood of CLL patients**

(A) Flow cytometric dot plots showing the presence of PD1$^{+}$BLIMP1$^{HI}$ T cells in CLL patients.

(B) CD3$^{+}$CD8$^{+}$PD1$^{+}$BLIMP1$^{HI}$ and (C) CD3$^{+}$CD4$^{+}$PD1$^{+}$BLIMP1$^{HI}$ cells are expanded in CLL patients.

### *3.4.4 Expression of CD127 (IL-7R) on T cells from CLL patients*

A further feature of exhausted T cells in many chronic viral infections is down-regulation of CD127 (IL-7R), as the T cells lose responsiveness to homeostatic cytokines and become dependent on antigen for continued survival (Paiardini *et al.* 2005; Radziewicz *et al.* 2007). There was a not statistically significant trend to decreased expression of CD127 on CLL $CD8^+$ T cells compared to healthy controls ($P$ = 0.079). However, there was a significant decrease in the expression of CD127 on CLL $CD4^+$ T cells ($P$ = 0.012)(**Figure 3.5**).

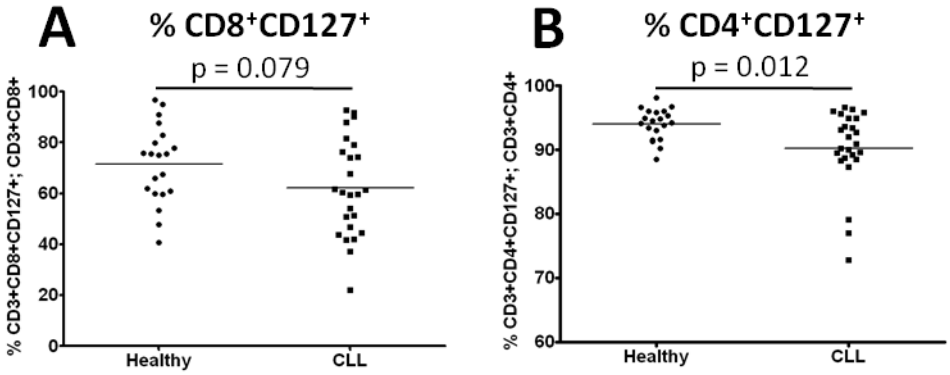



**Figure 3.5 Expression of CD127 on T cells from CLL patients**

Expression of CD127 (IL-7R) on $CD3^+CD8^+$ T cells (A) and $CD3^+CD4^+$ T cells (B).

### *3.4.5 CD8$^+$ T cells from patients with CLL show functional defects in proliferation*

T cell exhaustion also results in progressive loss of T-cell function, including loss of proliferative and cytotoxic capacity (Wherry 2011). Therefore, the ability of T cells from CLL patients and controls to proliferate in response to 72 hours of anti-CD3/anti-CD28 stimulation was assessed using a CFSE based assay (Lyons and Parish 1994). The proliferation index of both CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells from CLL patients was significantly lower than healthy controls (both: $P < 0.0001$)(**Figure 3.6**). There was also a reduction in the proportion of CD3$^+$CD8$^+$ T cells that had entered cell division, but no significant difference when comparing healthy and CLL CD3$^+$CD4$^+$ T cells. This suggests that the proliferative defect seen in CLL T cells is due to a combination of a reduction in the proportion of cells able to divide upon polyclonal activation, and prolongation of the division time of the proliferating cells.

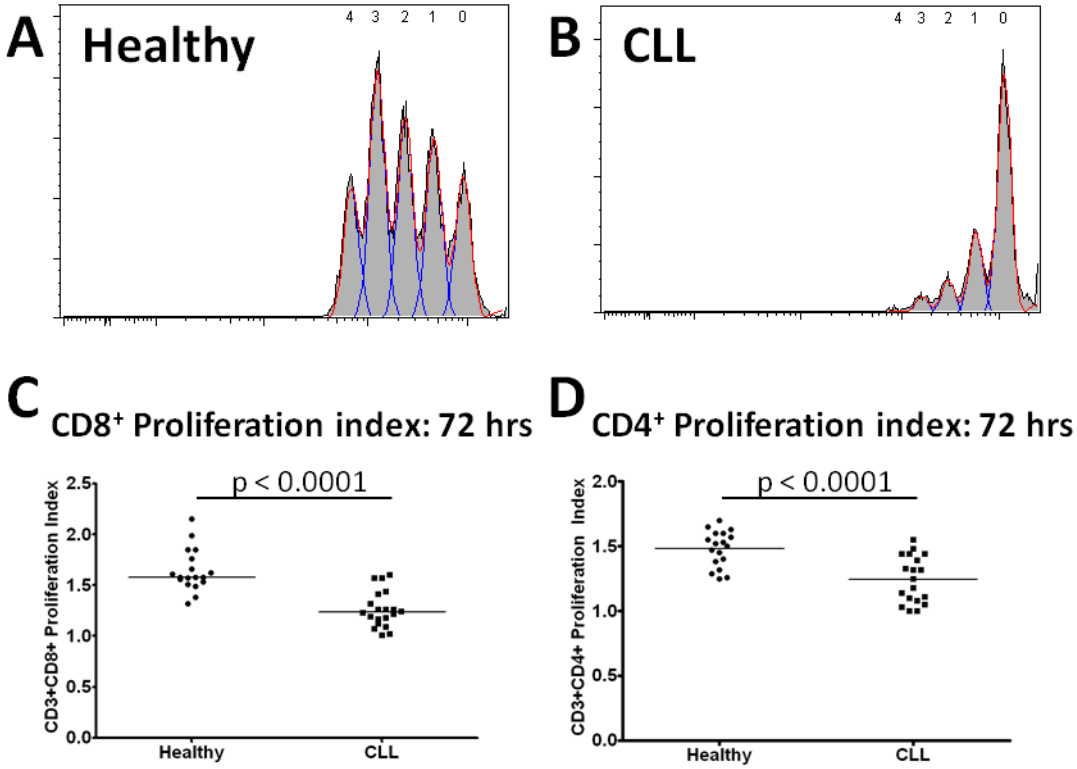



**Figure 3.6 T cells from CLL patients show functional defects in proliferation**

The proliferative potential of T cells from CLL patients and healthy controls was assessed using a CFSE based assay. Representative histograms are shown of (A) a healthy donor and (B) a CLL patient. (C) The proliferation index was significantly lower for CD3$^+$CD8$^+$ T cells from CLL patients when compared with healthy controls. (D) There was also a reduction in the proliferation index for CD3$^+$CD4$^+$ T cells from CLL patients when compared with healthy controls.

### *3.4.6 CD8$^+$ T cells from patients with CLL show functional defects in cytotoxicity*

Another feature of functional T-cell exhaustion is impairment of *ex vivo* target cell killing (Wherry *et al.* 2003). Therefore, the ability of CD8$^+$ T cells from CLL patients and healthy donors to lyse idiotype-pulsed target cells was assessed by a $^{51}$chromium release assay. In keeping with previous observations by my group, there was impaired ability to induce idiotype-specific CD8$^+$ T cells capable of killing idiotype-pulsed target cells compared to healthy donors (Krackhardt *et al.* 2002; Ramsay *et al.* 2008). This defect in target cell lysis was observed at all effector:target cell ratios. ($P < 0.0001$ at effector:target ratio of 30:1)(**Figure 3.7**).

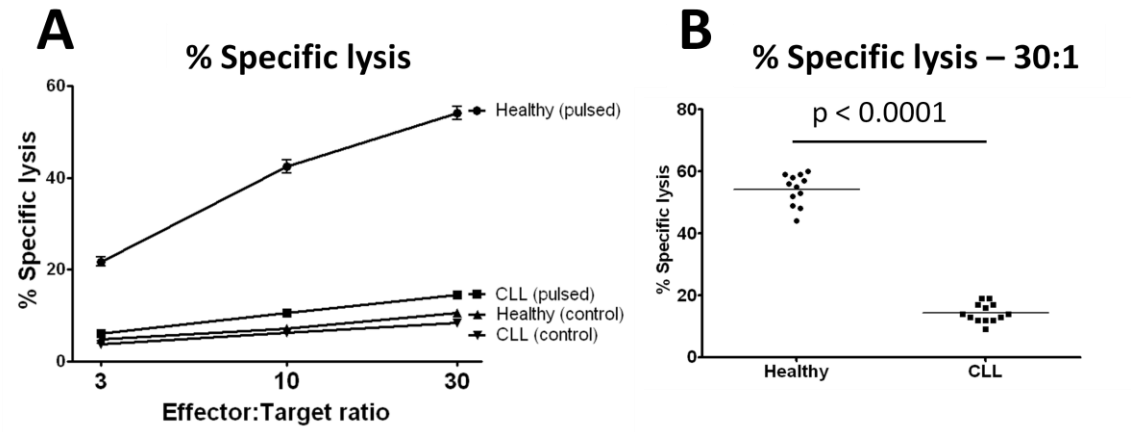



**Figure 3.7 CD8$^+$ T cells from CLL patients show functional defects in cytotoxicity**

The cytotoxic activity of CD8$^+$ T cells from patients and controls was assessed. CD8$^+$ T cells from CLL patients fail to lyse idiotype pulsed target cells in contrast to CD8$^+$ T cells from healthy controls: % specific lysis at all effector target ratios (A), and at a effector-target ratio of 30:1 (B). **Data generated in conjunction with Professor John Gribben and Dr Alan Ramsay.**

### *3.4.7 T cells from CLL patients retain the capacity for cytokine secretion*

A further feature of functional T-cell exhaustion during chronic viral infections is failure to produce effector cytokines. This happens in a hierarchical manner, with the ability to produce IL2 being lost at early stages of exhaustion, followed by loss of TNFα production and finally IFNγ (Shin and Wherry 2007). Therefore cytokine production after stimulation with PMA and ionomycin was analysed. In contrast to the pattern observed in chronic viral infections, $CD3^+CD8^+$ T cells from CLL patients had increased production of IFNγ ($P < 0.0001$) and TNFα ($P = 0.033$) without any reduction in IL2 production ($P = 0.29$) when compared with healthy controls. $CD3^+CD4^+$ T cells from CLL patients also showed increased production of IFNγ ($P = 0.0089$), along with increased IL2 ($P = 0.015$), and no reduction in TNFα production ($P = 0.20$)(**Figure 3.8**).

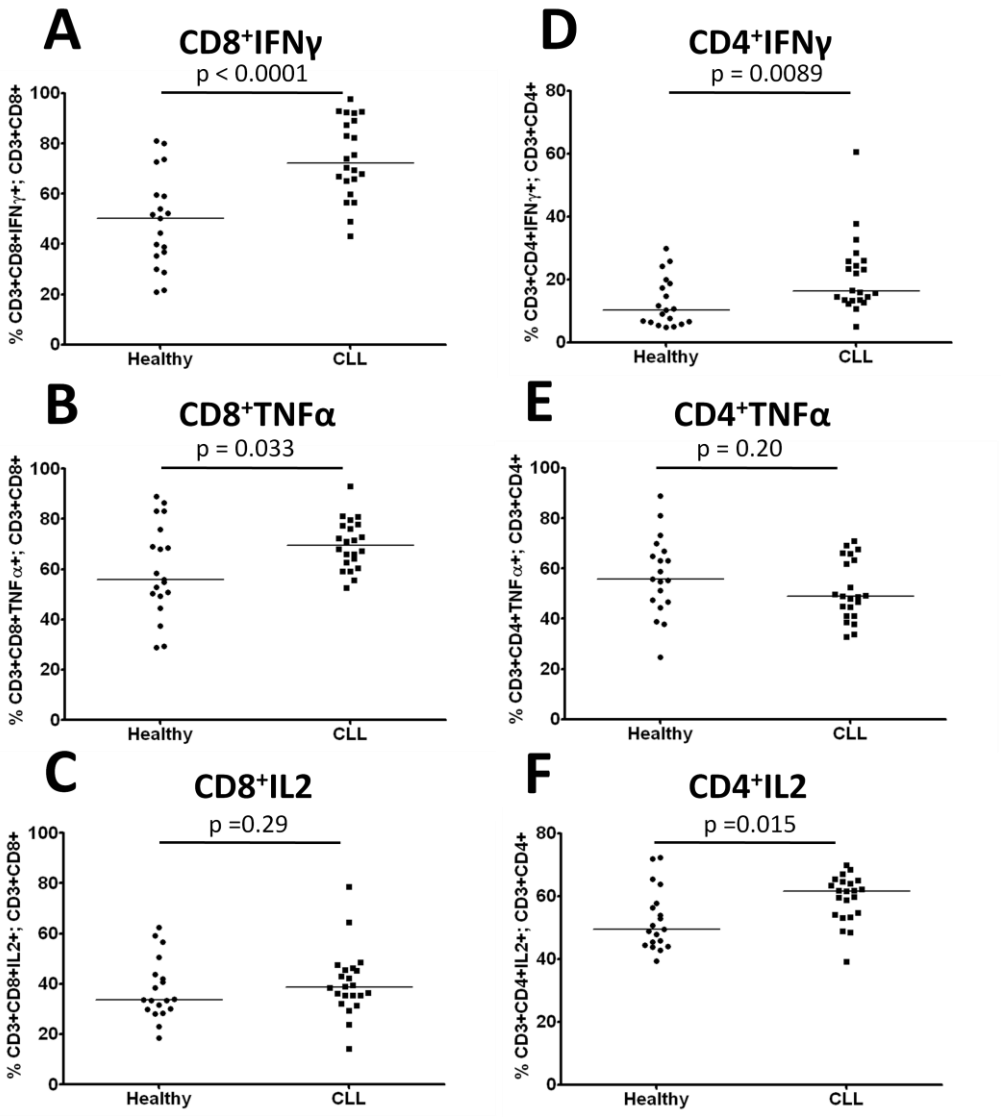



**Figure 3.8 Comparison of cytokine production by $CD8^+$ and $CD4^+$ T cells from CLL patients and healthy controls**

### *3.4.8 T cells from CLL patients have increased expression of TBET*

The transcription factor TBET plays a crucial role in T-cell development, acting as a master controller of Th1/Tc1 differentiation (Szabo *et al.* 2000). Given the higher production of IFNγ by both $CD8^+$ and $CD4^+$ T cells from CLL patients, it was postulated that they would have increased expression of TBET consistent being Th1/Tc1 differentiated. When TBET expression was assessed, both $CD3^+CD8^+$ T cells ($P = 0.0002$) and $CD3^+CD4^+$ T cells ($P < 0.0001$) had significantly higher expression than healthy T cells, providing support for this hypothesis.

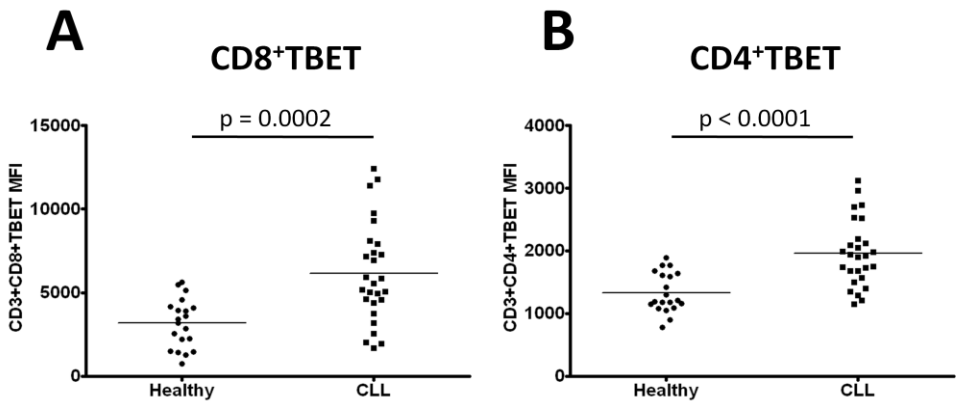



**Figure 3.9 T cells from CLL patients have increased expression of TBET**

### *3.4.9 CCR7⁻ effector T cells are increased in CLL*

Previous studies have shown skewing of the T-cell compartment towards activated T-cell subsets, in both CLL patients and in the Eμ-TCL1 mouse model.(Van den Hove *et al.* 1998; Hofbauer *et al.* 2011) Expression of C-C chemokine receptor type 7 (CCR7), a chemokine receptor that controls homing to secondary lymphoid organs, divides human memory T cells into two functionally distinct subsets. $CCR7^-$ cells express receptors for migration to inflamed tissues and display immediate effector function, whereas $CCR7^+$ cells express lymph-node homing receptors and lack immediate effector function, but efficiently stimulate dendritic cells and differentiate into $CCR7^-$ effector cells upon secondary stimulation (Sallusto *et al.* 1999). In light of this the balance between the $CCR7^+$ and $CCR7^-$ subtypes was investigated. When compared with healthy controls, there was both a greater proportion and increased numbers of $CD3^+CD8^+CCR7^-$ ($P$ = 0.0043) and $CD3^+CD4^+CCR7^-$ ($P < 0.0001$) T cells in CLL patients (**Figure 3.10**).

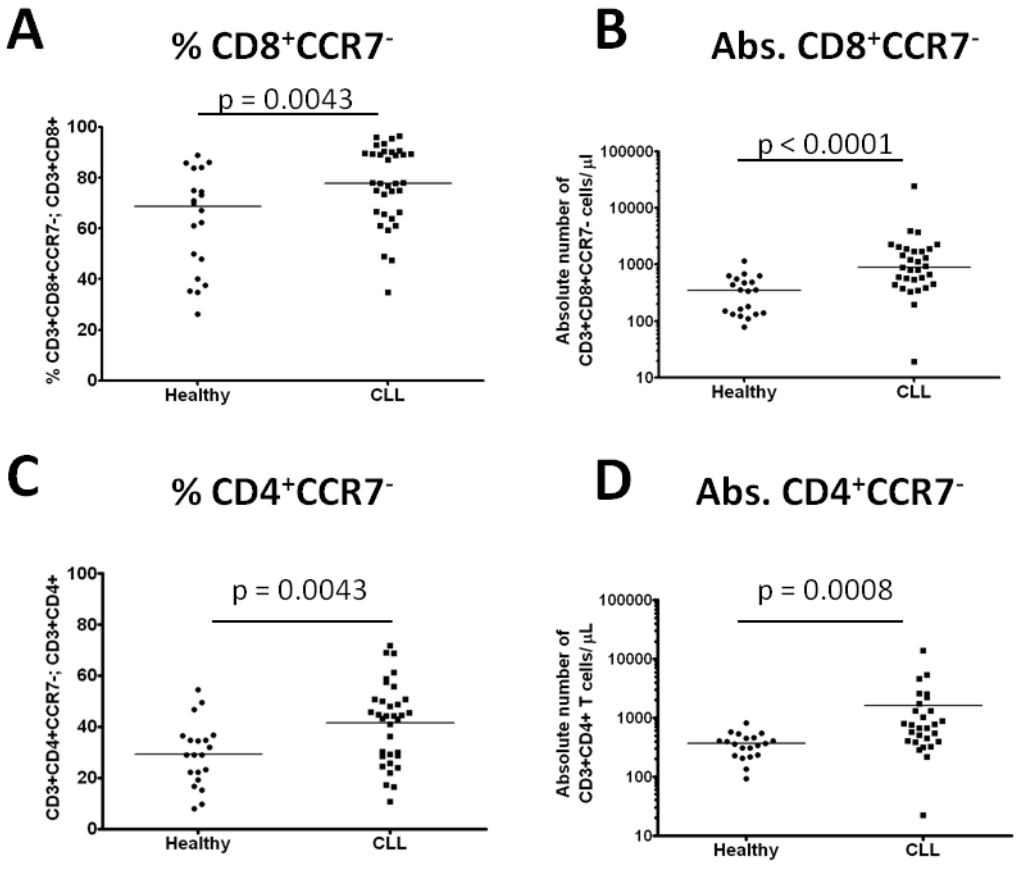



**Figure 3.10 $CCR7^-$ effector T cells are increased in CLL patients**

The expression of the chemokine receptor CCR7 was assessed. (A) & (B) CLL patients had an increased proportion and increased numbers of $CD3^+CD8^+CCR7^-$ T cells. (C) & (D) Similarly, CLL patients had an increased proportion and increased numbers of $CD3^+CD4^+CCR7^-$ T cells.

### *3.4.10 The observed phenotypic and functional changes in T cells from CLL patients reflect the skew towards CCR7⁻ effector subsets*

T cells can be further subdivided based on their expression of CD45RA, and defined as naïve ($CCR7^+CD45RA^+$), central memory ($T_{CM}$: $CCR7^+CD45RA^-$), and effector memory ($T_{EM}$: $CCR7^-CD45RA^-$). Within the $CD3^+CD8^+$ $T_{EM}$ subtype, there is also a subset that re-expresses CD45RA, termed $T_{EMRA}$-cells; this subset is rare within the $CD3^+CD4^+$ compartment (Sallusto *et al.* 2004). $T_{EMRA}$ cells have many similarities to exhausted T cells, in that they show reduced expression of co-stimulatory molecules such as CD28, reduced proliferative capacity, and an increased tendency to undergo apoptosis (Geginat *et al.* 2003). Therefore the phenotypic and functional parameters described above were correlated with the different $CD3^+CD8^+$T cell subsets as defined by their expression of CCR7 and CD45RA (**Figure 3.11;** summarised in **Table 3.2**). $T_{EMRA}$ cells showed many features consistent with exhaustion, with the highest expression of CD160, CD244 and TBET, the lowest expression of CD28, the lowest proliferative capacity, and loss of IL2 production. Notably however, $CCR7^-CD45RA^-$ $T_{EM}$ cells had the highest expression of PD1. These results indicate that the phenotypic and functional changes in T cells from CLL patients reflect the skew towards $CCR7^-$ antigen experienced effector subsets.

**Table 3.1 Summary of phenotypic and function parameters by $CD3^+CD8^+$ T subset**

| Subset | Naïve $CCR7^+CD45RA^+$ | Central Memory $CCR7^+CD45RA^-$ | Effector Memory $CCR7^-CD45RA^-$ | $T_{EMRA}$ $CCR7^-CD45RA^+$ |
|---|---|---|---|---|
| **PD1 expression** | Low | Low | High | Moderate |
| **CD160 expression** | Low | Low | Moderate | High |
| **CD244 expression** | Low | Low | Moderate | High |
| **CD28 expression** | High | High | Moderate | Low |
| **TBET expression** | Low | Low | Moderate | High |
| **EOMES expression** | Low | Low | High | High |
| **IFNγ production** | Low | Moderate | High | High |
| **IL2 production** | Low | High | Moderate-High | Moderate |
| **TNFα production** | Low | High | High | Moderate-High |
| **Proliferative capacity** | High | High | Moderate | Low |

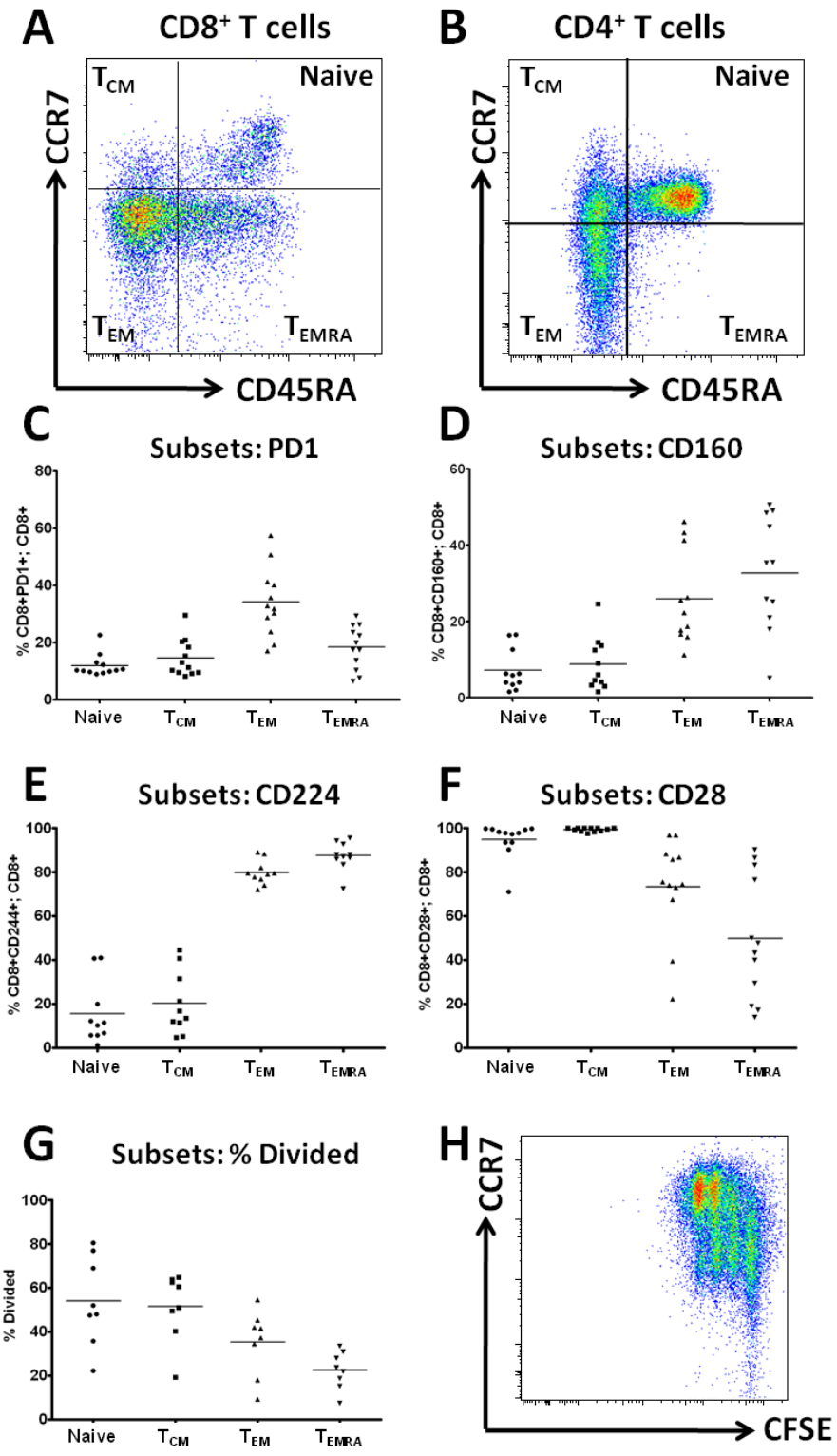



**Figure 3.11 Phenotypic and functional parameters by $CD3^+CD8^+$ subsets**

(A) Within the $CD3^+CD8^+$ compartment all 4 subsets were observed. (B) In comparison $CCR7^-$ $CD45RA^+$ cells were rarely seen in the $CD3^+CD4^+$ compartment. (C) Expression of PD1 was highest on $T_{EM}$ cells. (D)&(E) In contrast, expression of CD160 and CD244 was increased on $T_{EM}$ cells, but was highest on $T_{EMRA}$ cells. (F) Expression of CD28 was decreased on $T_{EM}$ cells, but was lowest on $T_{EMRA}$ cells. (G) The proliferative capacity of $T_{EM}$ cells was reduced, but $T_{EMRA}$ cells had the lowest capacity to proliferate. (H) Flow cytometric dot plot showing the relative reduction in proliferative capacity (CFSE dye dilution) of $CD3^+CD8^+CCR7^-$ T cells compared to $CD3^+CD8^+CCR7^+$ T cells.

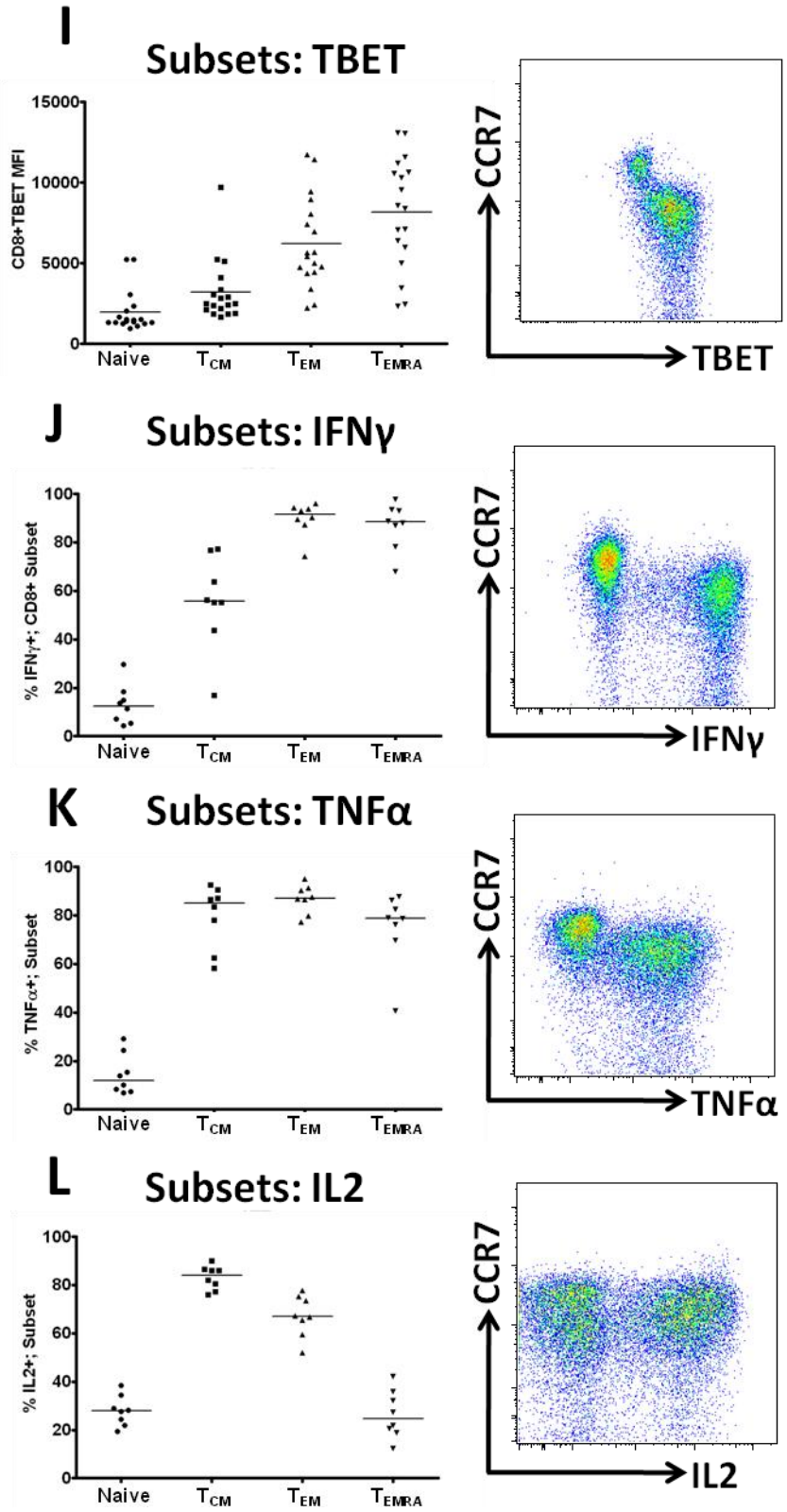



**Figure 3.11 (continued)**

The expression of TBET and the production of IFNγ, TNFα, and IL2 by $CD3^{+}CD8^{+}$ T-cell subsets from CLL patients was analysed. (I) The expression of TBET was increased in $T_{EM}$ cells, with the highest expression levels in the $T_{EMRA}$ subset. (J) – (L) $T_{EMRA}$ cells had high production of IFNγ, slightly reduced production of TNFα, and substantially reduced production of IL2.

### *3.4.11 CD8$^+$ T cells from CLL patients have increased expression of eomesodermin*

Eomesodermin (Eomes) is a second transcription factor implicated in both effector differentiation and T-cell exhaustion. Eomes has been shown to be upregulated in exhausted CD8$^+$ T cells, with Eomes-expressing cells having higher expression of Blimp-1 and several inhibitory receptors including PD1 (Paley *et al.* 2012). Therefore, the expression of Eomes in T cells from healthy donors and CLL patients was assessed. CD3$^+$CD8$^+$ T cells from CLL patients had significantly higher expression of Eomes than healthy CD3$^+$CD8$^+$ T cells ($P$ = 0.0061), whereas there was no difference in the CD3$^+$CD4$^+$ compartment. The expression of Eomes was also assessed in the various CD3$^+$CD8$^+$T cell subsets as defined by expression of CCR7 and CD45RA. Expression of Eomes was confined to the CCR7$^-$ subsets, $T_{EM}$ and $T_{EMRA}$ (**Figure 3.12**).

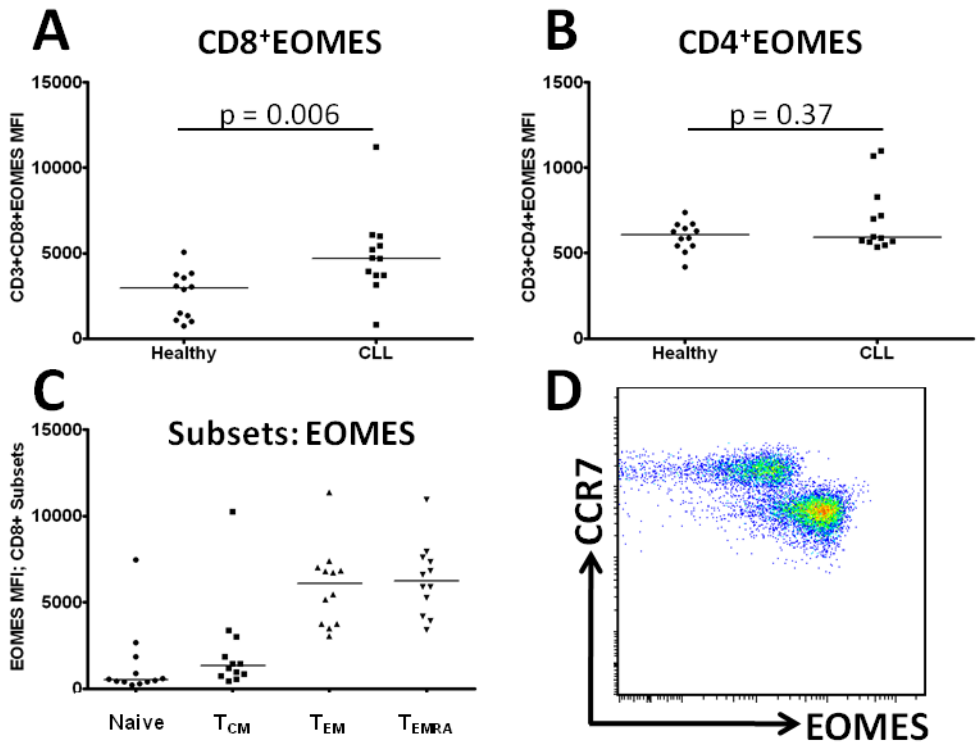



**Figure 3.12 CD8$^+$ T cells from CLL patients have increased expression of Eomes**

The expression of Eomes was compared in healthy and CLL T cells. (A CD3$^+$CD8$^+$ T cells from CLL patients had significantly higher expression of Eomes than healthy T cells. (B) No difference was observed when comparing healthy and CLL CD3$^+$CD4$^+$ T cells. (C) & (D) Expression of Eomes was largely confined to CCR7$^-$ effector subsets.

### *3.4.12 The impact of CMV serostatus on expression of CD244, CD160, and PD1*

CMV seropositivity is known to have a profound influence on the major lymphoid subsets in healthy individuals, with expanded populations of CMV specific $CD4^+$ and $CD8^+$ T cells reported in CMV+ CLL patients (Khan *et al.* 2002; Mackus *et al.* 2003; Chidrawar *et al.* 2009; Pourgheysari *et al.* 2010). Since T-cell exhaustion has been extensively described in chronic viral infections, it was important to assess the effect of this in CLL. Expression of CD244 and CD160 was increased on $CD3^+CD8^+$ T cells from CLL patients compared to healthy controls irrespective of CMV-serostatus. In contrast, PD1 expression was increased on CLL $CD3^+CD8^+$ T cells only when comparing CMV- individuals ($P = 0.002$). The increased expression of CD244, CD160, and PD1 seen in CLL patients again reflected the skew towards $CD3^+CD8^+CCR7^-$ effector cells, which were expanded in CLL patients irrespective of CMV-serostatus. Qualitatively similar findings were observed in the $CD3^+CD4^+$ compartment, although the expression of CD244, CD160, and PD1 was considerably lower $CD3^+CD4^+$ T cells, and the differences were not always statistically significant. One important area where the expression of these molecules did differ when comparing $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells, was that expression of PD1 was increased on $CD3^+CD4^+$ T cells from CLL patients irrespective of CMV serostatus (**Figure 3.13**).

To examine these findings in more detail, the proportion of naïve, $T_{CM}$, $T_{EM}$ and $T_{EMRA}$ cells was compared in CLL patients and controls separately for CMV+ and CMV- individuals. Importantly, CMV serostatus affected the balance of the $T_{EM}/T_{EMRA}$ subsets, with CMV seropositivity skewing $CD8^+$ T-cell differentiation from a naïve to a $T_{EMRA}$ phenotype in both patients and healthy controls. In contrast, in CMV- CLL patients, the increased proportion of $CD8^+CCR7^-$ effector cells primarily reflected an expansion of $T_{EM}$ cells. These alterations in the distribution of lymphocyte subsets account for the observation that PD1 expression was only significantly increased in CMV- CLL patients, as the expanded $T_{EM}$ cells seen in these patients had increased expression of PD1. In contrast, in CMV+ CLL patients there was no global increase in PD1 expression, since there was expansion of $T_{EMRA}$ cells that have lower expression of PD1. Expression of PD1 was increased on $CD3^+CD4^+$ T cells from CLL patients irrespective of CMV serostatus, as the equivalent $T_{EMRA}$ subset is not present in the $CD3^+CD4^+$ compartment as described above (**Figure 3.14**).

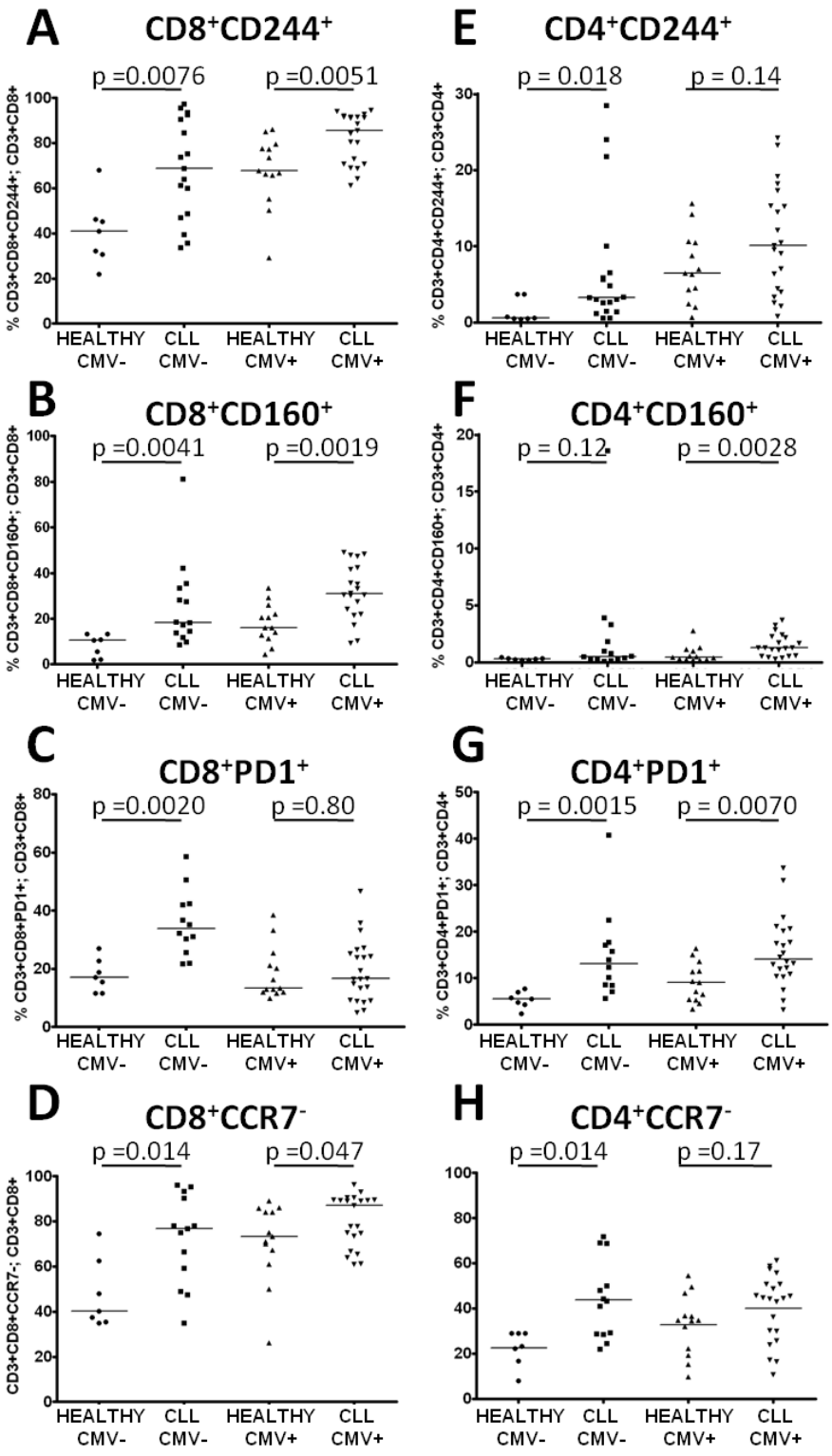



**Figure 3.13 The impact of CMV serostatus on T-cell phenotype**

Significant increases in the expression of CD244 (A) and CD160 (B) on CLL $CD3^+CD8^+$ T cells were observed independently of CMV serostatus. (C) In contrast, expression of PD1 was only increased when comparing CMV- patients and controls. (D) $CD3^+CD8^+CCR7^-$ cells are expanded in CLL irrespective of CMV serostatus. (E) & (F) Similar differences in the expression of CD244 and CD160 were noted on $CD3^+CD4^+$ T cells although the level of expression was considerably lower. (G) In contrast, expression of PD1 $CD3^+CD4^+$ T cells was increased in CLL patients irrespective of CMV- serostatus. (H) $CD3^+CD4^+CCR7^-$ cells are expanded when comparing CMV- patients and controls.

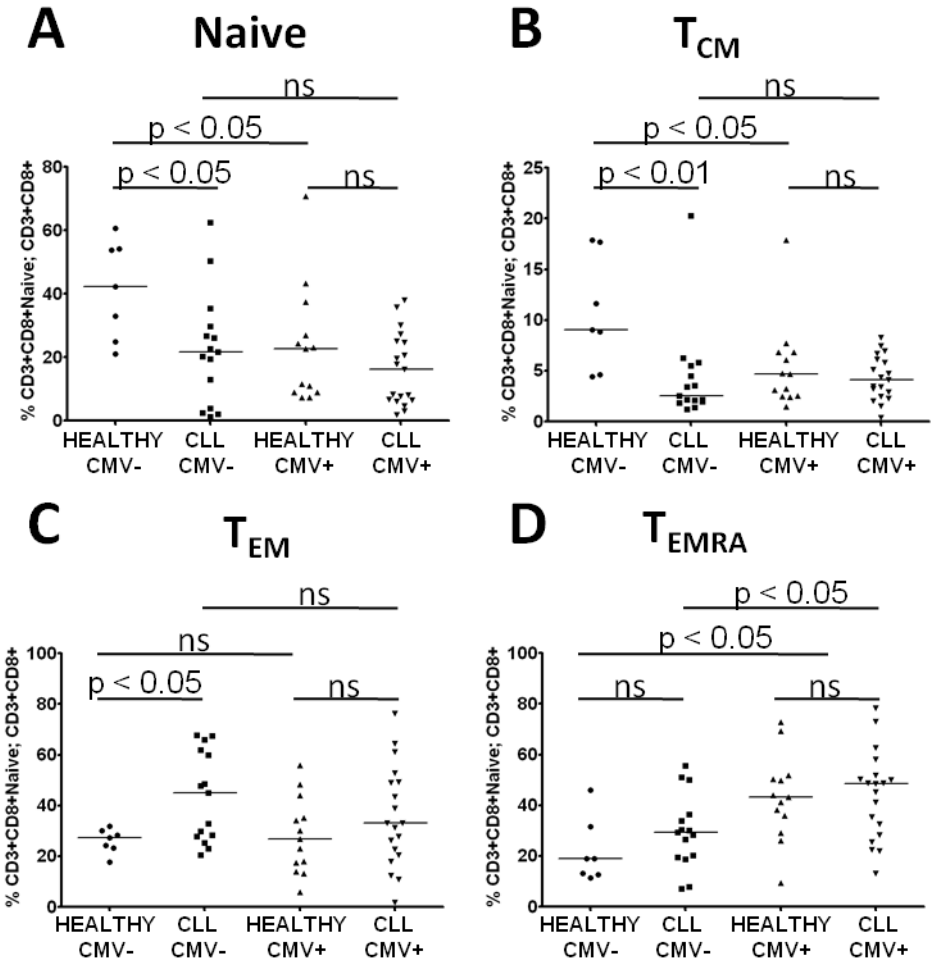



**Figure 3.14 CLL skews the $CD3^+CD8^+$ T cell repertoire towards a $CCR7^-CD45RA^-$ effector memory phenotype, whereas the presence of CMV leads to an expansion of $CCR7^-CD45RA^+$ $T_{EMRA}$ subtype**

CLL patients and healthy CMV+ individuals had a reduction in $CCR7^+CD45RA^+$ naive (A) and $CCR7^+CD45RA^-$ central memory T-cell (B) numbers when compared with healthy CMV- controls. (C) $CCR7^-CD45RA^-$ effector memory T cells were only significantly expanded when comparing CMV- patients and controls. (D) In contrast $CCR7^-CD45RA^+$ $T_{EMRA}$ cells were only significantly expanded in CMV+ patients and controls.

### *3.4.13 The defects in CLL T-cell function are present irrespective of CMV*

The description of expanded populations of CMV-specific T cells in CLL also raised the question as to whether the observed defects in T-cell function are due to CMV (Akbar 2011). Therefore the impact of CMV serostatus on proliferation, cytolytic activity, and cytokine production of T cells from CLL patients was examined. The proportion of $CD3^+CD8^+$ T cells that were positive for IFNγ was increased in CLL patients irrespective of CMV serostatus, correlating with the expression of TBET. A similar pattern was observed in the $CD3^+CD4^+$ compartment. Both $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells from CLL patients also exhibited a decreased capacity to proliferate irrespective of CMV serostatus. The ability of $CD8^+$ T cells from CLL patients to lyse idiotype pulsed target cells was assessed. There was a reduction in the cytolytic function of $CD3^+CD8^+$ T cells from CLL patients in all cases, regardless of CMV serostatus. These findings exclude CMV seropositivity as the sole cause of functional defects in CLL T cells.

**Figure 3.15 (overleaf) The defects in T-cell function observed in CLL are present irrespective of CMV serostatus**

The function of CLL T cells was compared with T cells from healthy controls matched for CMV serostatus. (A) Increased production of IFNγ by CLL $CD3^+CD8^+$ T cells was observed irrespective of CMV serostatus. (B) Increased production of IFNγ by CLL $CD3^+CD4^+$ T cells was observed when comparing CMV- individuals; there was a trend to increased production when comparing CMV+ individuals but this was not statistically significant. (C) & (D) There was increased expression of TBET in $CD3^+CD8^+CCR7^-$ and $CD3^+CD4^+CCR7^-$ T cells from CLL patients irrespective of CMV serostatus. (E) & (F) $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells from CLL patients showed decreased proliferative capacity irrespective of CMV serostatus. (G) A reduction in the cytolytic capacity of $CD3^+CD8^+$ T cells was also observed in all CLL patients. Pulsed target cells (closed symbols); unpulsed control target cells (open symbols). The graph representing % specific lysis shows the mean and standard error of results obtained from 13 CLL patients, (5 CMV- and 8 CMV+), and 12 healthy controls (5 CMV- and 7 CMV+).

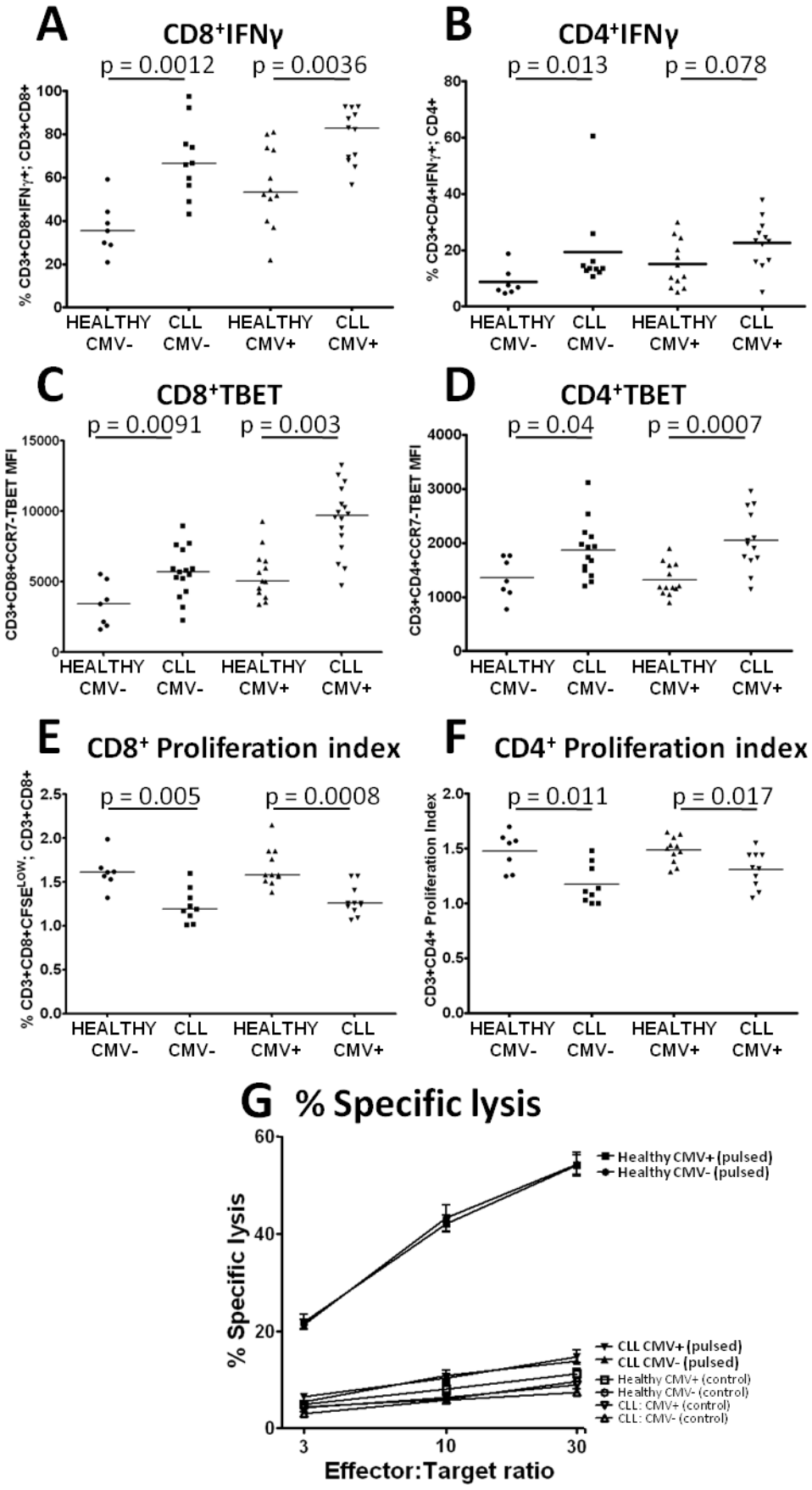



**Figure 3.15 The defects in T-cell function observed in CLL are present irrespective of CMV serostatus**

### *3.4.14 CD8$^+$ T cells from CLL patients show defective cytotoxicity due to failure of granzyme localization to the immunological synapse*

My group has previously demonstrated that CLL T cells exhibit impaired actin polymerization and defective immunological synapse formation with APCs. Therefore, the impact of dysfunctional immunological synapse formation on the observed cytolytic defects was examined. The ability of the CD8$^+$ T cells to degranulate in response to SEB was assessed by analysis of CD107a (LAMP1) transfer to the cell surface (Betts *et al.* 2003). CD8$^+$ T cells from CLL patients retained the ability to degranulate, and actually showed enhanced transfer of CD107a to the cell surface, again irrespective of CMV serostatus. Confocal microscopic analysis revealed that while normal T cells were able to localize F-actin, CD107a and granzyme B to the immunological synapse, there was failure of transport of these molecules within CLL T cells, irrespective of their level of expression. This suggests that the cytotoxic defect seen in CLL T cells is due to a combination of disordered, non-polarized degranulation, and failure of effective granzyme B packaging into the cytolytic vesicles (**Figure 3.16**).

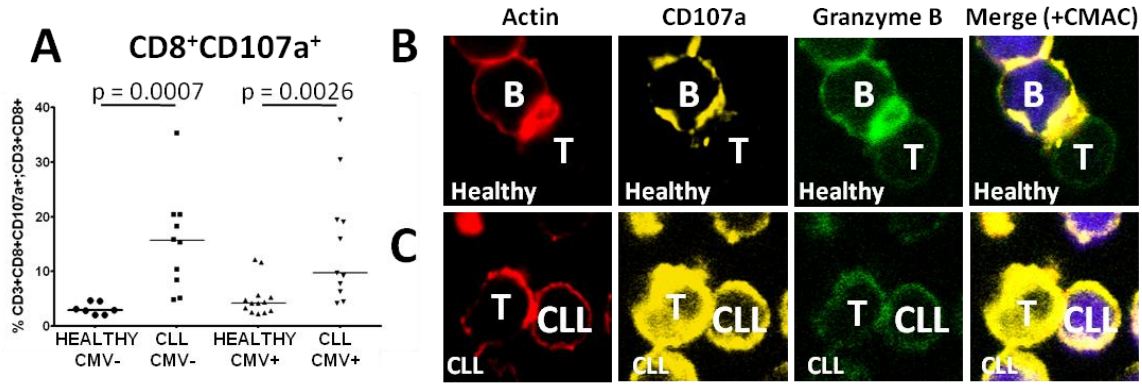



**Figure 3.16 CD8$^+$ T cells from CLL patients show defective cytotoxicity due to failure of granzyme localization to the immunological synapse**

The cytotoxic activity of CD8$^+$ T cells from CLL patients was assessed further. (A) CD8$^+$ T cells from CLL patients retain the ability to degranulate in response to SEB, as shown by their increased ability to transfer CD107a to the cell surface. (B) Healthy CD8$^+$ T cells showed effective F-actin (red, rhodamine phalloidin) immunological synapse formation with co-localization of CD107a (yellow, Alexa Fluor 647) and granzyme B (green, Alexa Fluor 488) at the synapse contact site with healthy B cells (+sAg). (C) In contrast, CD8$^+$ T cells from CLL patients fail to form effective F-actin immune synapses with autologous CLL cells (+sAg) that is associated with strong, non-polarized expression of CD107a and a lack of granzyme B polarization to the contact site.

## 3.5 Discussion

This study demonstrates that T cells from CLL patients exhibit features of T-cell exhaustion, an acquired state of T-cell dysfunction that was first described in the context of chronic viral infections (Shin and Wherry 2007). Both $CD4^+$ and $CD8^+$ T cells are expanded in the peripheral blood of CLL patients, with the degree of expansion being greater in the $CD8^+$ compartment, consistent with previous reports (Lauria *et al.* 1980; Herrmann *et al.* 1982; Platsoucas *et al.* 1982). CLL T cells have increased expression of the exhaustion markers, CD244, CD160, and PD1, and show functional evidence of exhaustion with an impaired capacity to proliferate. $CD8^+$ T cells also have a cytotoxic defect, having a reduced ability to lyse target cells. These phenotypic and functional defects are more apparent in $CCR7^-$ effector cells, and the global defects described here correlate with a skewing of T-cell subsets in the peripheral blood of CLL patients towards effector differentiation.

These results also provide evidence that the transcriptional control of T-cell differentiation is also abnormal in CLL, with increased expression of BLIMP1, TBET, and Eomes. Our understanding of the role of these transcription factors in regulating both normal T-cell differentiation and T-cell exhaustion is still evolving. A role for both TBET and Eomes in promoting the normal differentiation of effector memory cells is well established, and it perhaps of no surprise that the expression of these transcription factors is increased in T cells from CLL patients, where there is skew towards effector differentiation (Sullivan *et al.* 2003; Intlekofer *et al.* 2005). However, the interplay of these molecules with other transcription factors such as BLIMP1, NFAT2, and BATF in the regulation of T-cell exhaustion is complex (Wherry 2011). It has recently emerged that BLIMP1 and Eomes are both implicated in promoting high expression of PD1, but TBET can actually suppress expression of this inhibitory receptor and sustain $CD8^+$ T-cell responses (Kao *et al.* 2011; Paley *et al.* 2012). These findings may explain the observations reported here that $T_{EM}$ cells have a higher expression of PD1 than $T_{EMRA}$ cells, which otherwise more classically phenotypically and functionally "exhausted". These results are consistent with descriptions of exhausted T cells in HIV infection, where HIV tetramer positive PD1-expressing cells had low expression of both CCR7 and CD45RA, consistent with a $T_{EM}$ phenotype (Day *et al.* 2006). The high expression of PD1 in $T_{EM}$ cells may be due to the activity of Eomes and BLIMP1 on a background moderate TBET expression. In contrast the relatively lower expression of PD1 on

$T_{EMRA}$ cells may be due to the very high expression of TBET, suppressing the expression of this inhibitory ligand.

The principal way in which CLL T cells differ from exhausted T-cells described in chronic viral infections is that they retain the capacity to produce cytokines such as IFNγ and TNFα. It is possible that the use of PMA and ionomycin to stimulate cytokine production could potentially overcome any subtle defects in cytokine production. However, a *greater* proportion of CLL T cells were positive for the Th1 cytokines IFNγ and TNFα, and this data correlated well with the expression of TBET, the prototypical Th1 transcription factor, that was analysed in resting cells (Szabo *et al.* 2000). Furthermore, there was relatively less production of IL2 in $T_{EMRA}$ cells, which had the highest expression of CD244 and CD160, and the lowest proliferative capacity. The observation of a relative preservation of cytokine production may reflect differences in antigen affinity, with chronic stimulation by high affinity viral antigens leading to a spectrum of functional disturbances distinct to that seen after chronic stimulation by low affinity self-antigens. It is also possible that the exhaustion pathways are co-opted by CLL cells to inhibit immune responses, but to maintain production of cytokines such as IFNγ and TNFα which have been shown to be pro-tumoural (Digel *et al.* 1989; Buschle *et al.* 1993). This finding may also be of immunotherapeutic importance, signifying a less exhausted state that could potentially be reversed with appropriate intervention.

Interestingly, the phenotypic and functional profile reported here mirrors that of "senescent" T cells induced by regulatory T cells (Ye *et al.* 2012). Regulatory T cells are expanded in CLL, with numbers correlating with disease stage and prognosis, so it is probable that this T-cell subgroup regulates anti-tumour immune responses in CLL (Beyer *et al.* 2005; Giannopoulos *et al.* 2008; D'Arena *et al.* 2012). Regulatory T cells are one of the three main pathways implicated in promoting the development of T-cell exhaustion in combination with chronic antigenic drive (Wherry 2011). The other two major pathways involve inhibitory cytokines such as IL10, and inhibitory signalling axes such as PD1:PDL1 interactions. Serum levels of IL10 are known to be increased in patients with CLL, with higher levels associated with impaired prognosis (Fayad *et al.* 2001). The role of inhibitory axes in CLL has been a target of recent research by my group. T cells from CLL patients exhibit impaired ability to form immunological synapses with antigen presenting cells, and this defect can be induced in healthy allogeneic T cells by co-culturing them with CLL cells (Ramsay *et al.* 2008).

Subsequent work demonstrated that this inducible defect was dependent on the expression of four inhibitory ligands expressed by CLL cells: CD200, CD270 (HVEM), CD274 (PDL1), and CD276 (Ramsay *et al.* 2012). Two of these molecules, PDL1 and HVEM are ligands for inhibitory receptors (PD1 and CD160) expressed by exhausted T cells, suggesting that PD1:PDL1 and CD160:HVEM interactions are crucial in promoting exhaustion and suppression of T-cell function in CLL. This may well be of importance in other cancers given recent reports documenting mutations/gene fusions of PDL1 and HVEM in other B-cell malignancies.(Cheung *et al.* 2010; Steidl *et al.* 2011)

Ever since the description of expanded populations of CMV specific T cells in CLL, it has been unclear as to the contribution of these populations to the T-cell defects observed in this disease (Mackus *et al.* 2003; Pourgheysari *et al.* 2010). It has been suggested that these CMV specific T cells inhibit the function of other antigen-specific T cell populations, either by a direct effect, or by constriction of the total T-cell repertoire (Akbar 2011). These results show that while CMV positivity modulated the distribution of lymphocyte subsets, the functional defects were present irrespective of CMV serostatus, thereby excluding CMV as the sole cause of T-cell defects in CLL. The modulation of lymphocyte subset distribution accounts for some previously reported differences regarding the expression of PD1 on CLL T cells (Nunes *et al.* 2012; Tonino *et al.* 2012). Use of both CMV serostatus and expression of CCR7/CD45RA allowed the impact of CLL and CMV on the distribution of $CD8^+$ T cell subsets to be disentangled. In agreement with previous studies, $T_{EMRA}$ cells were only significantly expanded in CMV+ patients and controls (Mackus *et al.* 2003; Tonino *et al.* 2012). However it was also observed that the presence of CLL alone skews the T cell repertoire towards a $T_{EM}$ phenotype. Interestingly, as described above, $T_{EMRA}$ cells are "classically exhausted", with the highest expression of CD244 and CD160, the lowest proliferative capacity, and loss of IL2 production. Both CLL and CMV exposure lead to a reduction in naive and $T_{CM}$ cell numbers compared with healthy CMV-controls.

Given the importance of BCR-signalling in CLL, there has been a great deal of speculation as to the identity of other antigens that may drive this disease (Rosen *et al.* 2010). A recent landmark paper has provided evidence that a particular feature of CLL BCRs is that they are able to induce autonomous signalling independent of antigen, in a manner dependent on the heavy-chain complementarity-determining region (HCDR3)

and an internal epitope of the BCR.(Minden *et al.* 2012) A further finding was that BCRs derived from leukemic cells in the TCL1 mouse model of CLL were also autonomously active. As described above, the development of leukaemia in this mouse model is also associated with an expansion of $CD8^+$ effector T cells, despite being dependent on the single TCL1 transgene. It is therefore possible that the "pseudoexhausted" state of CLL T cells described here is due to their chronic stimulation by autonomously active CLL cells. However, several other reports have implicated a role for a wide variety of specific antigens in CLL, so this remains a controversial area.

In conclusion, this work has identified a novel phenotypic and functional profile of terminally differentiated $CD8^+$ T cells in patients with CLL. These "pseudo-exhausted" T cells may be a result of chronic stimulation by low affinity self-antigens, producing a state that is distinct to the exhaustion seen after chronic stimulation by high affinity viral antigens. They retain some functions, such as the ability to produce pro-tumoural cytokines and the ability to degranulate, but their effector cytotoxic function is blocked by cytoskeletal defects. These are likely to be due to a combination of their pseudo-exhausted state, and increased interaction with inhibitory ligands expressed on the surface of the CLL cells (Wherry *et al.* 2007; Ramsay *et al.* 2012). Furthermore, although CMV seropositivity has profound effects on the T-cell repertoire of both healthy individuals and CLL patients, these alterations in T-cell phenotype and function are also apparent in CMV- patients.

There do remain some answered questions. Much of the groundwork in understanding T-cell exhaustion has been undertaken in $CD8^+$ T cells, with this phenomenon being less well characterised in $CD4^+$ T cells. Therefore it remains unclear as to what the implications of this area are for the lymph node microenvironment in CLL, where $CD4^+$ T cells appear to be particularly important in promoting tumour cell survival (Pizzolo *et al.* 1983; Bagnara *et al.* 2011). Furthermore, T-cell exhaustion is thought to be chronic process that occurs over weeks/months, yet functional defects can be induced in healthy T cells after only 24 hours in co-culture with CLL cells. However, these results do have potential implications for immunotherapy, as molecules such as CD244, CD160 and PD1 may be useful biomarkers of immune reconstitution, and be potential targets for therapeutic strategies aimed at improving T-cell immunity. Interestingly, the immunomodulatory drug lenalidomide is able to repair many of the functional defects

associated with T-cell exhaustion. Data from pre-clinical models and clinical trials has shown that lenalidomide treatment is able to enhance cytokine production, improve proliferative capacity, and repair immunological synapse formation and cytotoxic function of CLL T cells (Corral *et al.* 1999; Ramsay *et al.* 2008; Shanafelt *et al.* 2013). Furthermore, lenalidomide is able to modulate "exhausting promoting pathways", by down-regulating the expression of inhibitory receptors and ligands, and by inhibiting the differentiation of regulatory T cells (Galustian *et al.* 2009; Idler *et al.* 2009; Ramsay *et al.* 2012). Investigation into how lenalidomide exerts these effects will be the focus of the next two chapters.

# Chapter 4: The Impact of L enalidomide on Gene Expression Profiling of Healthy and CLL lymphocytes

## 4.1 Introduction

Lenalidomide has recently been demonstrated to have significant activity in CLL. As it is not directly toxic to tumour cells *in vitro,* this clinical effect appears to be mediated by improvement of anti-tumour immune responses and reduction of pro-tumoural factors in the CLL microenvironment. An interesting feature of its clinical use is a "tumour flare reaction" which is associated with features of both CLL-cell and T-cell activation (Aue *et al.* 2009). There is some evidence that the presence of a tumour flare reaction correlates with improved outcome, but this is controversial and confounded by the use of prophylactic anti-inflammatory agents and by the use of highly immuno-suppressive drugs as part of previous treatments. An effect on NK cells also appears to contribute to the clinical efficacy of this agent, as the presence of higher numbers of NK-cells pre-treatment and better NK-cell function, predicted for better outcome in a clinical trial (Chanan-Khan *et al.* 2011; Chanan-Khan *et al.* 2011). However, the exact mechanism of action of this agent in CLL in particular how it repairs anti-tumour immune responses, remains unclear. An improved understanding of lenalidomide's mechanism of action in CLL could provide novel insights into the nature of the immune defect in this disease.

My group previously demonstrated profound changes in the global gene expression profiles of T cells from CLL patients when compared with healthy age-matched controls (Gorgun *et al.* 2005). Despite not being part of the malignant clone, alterations were found in the expression of genes involved in cell differentiation and cytoskeletal formation in patient $CD4^+$ T cells, and in cytoskeletal formation, vesicle trafficking, and cytotoxicity pathways in patient $CD8^+$ T cells. They subsequently showed that these changes in gene expression translate into a deficit in T-cell function, due to impaired actin polymerization resulting in defective immunological synapse formation. Treatment of both autologous T-cells and CLL cells with lenalidomide was able to repair this defect, suggesting that this may be a key component of this agent's activity in CLL (Ramsay *et al.* 2008).

## 4.2 Aims and objectives

In light of the fact that the cytoskeletal defect was initially observed as a consequence of comparing the gene expression profiles of T cells from CLL patients and healthy donors, we hypothesised that investigation of the impact of lenalidomide on the transcriptome of T cells would lead to novel mechanistic insights. Given that CLL-cell and NK-cell activation has also been implicated in the clinical efficacy of lenalidomide, we also aimed to investigate the impact of this agent on the transcriptome of CLL cells and NK cells. As my group and others have previously reported the alterations in the gene expression profiles of CLL B and T cells when compared with healthy lymphocytes, we also aimed to examine the effect of lenalidomide on these subsets from healthy donors to investigate whether there was a differential effect. And finally, a comparison of the gene expression profiles of NK cells from CLL patients and healthy controls has never been reported: a further aim was to investigate this.

## 4.3 Materials and Methods

### *4.3.1 Patients and Samples*

For the gene expressing profiling, peripheral blood samples were obtained from 22 CLL patients from the tissue bank maintained by the Department of Haemato-Oncology of St. Bartholomew's Hospital, London. Ethical approval was confirmed by the East London & The City Health Authority Local Research Ethics Committee, and written informed consent was obtained in accordance with the Declaration of Helsinki. All of the patients were untreated at time of blood withdrawal, and had a median age of 60 years (range 43 – 84). The patients had predominantly early stage CLL with 18/22 (81.8%) classed as having Binet stage A disease. Healthy PBMCs were obtained from 16 buffy coats/cones supplied by the National Blood Service. For the validation experiments, a second cohort of patient samples was obtained from a combination of Bart's Haemato-Oncology tissue bank and the CLL Research Consortium tissue core.

### *4.3.2 Preparation for Cell Culture and Lenalidomide Treatment*

Peripheral blood samples were diluted 1:1 with PBS prior to separation of PBMCs by density gradient centrifugation as described in section 2.4. Buffy coats/cones were diluted 4:1 with PBS due to the higher concentration of mononuclear cells. After density gradient centrifugation up to 1 x $10^9$ PBMCs were split into two fractions for cell culture. CLL or healthy PBMCs were then cultured at a concentration of 5 x $10^6$/ml for 24-48 hours with either 1μM lenalidomide or vehicle control. As the lenalidomide has been reconstituted in DMSO, 0.01% DMSO (Fisher Scientific) was added as the vehicle control.

### *4.3.3 Isolation of lymphocyte subsets*

After 48 hours the healthy or malignant B and T cells were separated using the AutoMACS Pro (Miltenyi Biotec) as described in section 2.5.3. B cells were positively selected using CD19 microbeads, whereas $CD4^+$ or $CD8^+$ T cells were negatively selected using the relevant isolation kits (Miltenyi Biotec). Due to the excess numbers of B cells in CLL PBMCs, the malignant B cells were pre-depleted prior to negative isolation of T or NK cells as described in section 2.5.4. Initial experiments showed that quantity and viability of NK cells decreased rapidly during the *in vitro* culture. In light of this, NK cells were isolated after 24 hours culture.

### *4.3.4 Determination of the purity of the isolated lymphocyte subsets*

After cell isolation a sample of the isolates was taken for determination of purity by flow cytometry. The purity of $CD4^+$ and $CD8^+$ T cells from both healthy and CLL patients was determined using antibodies to detect CD3, and CD4 and CD8 respectively. The purity of NK cells from both healthy and CLL patients was determined using antibodies to detect CD3 and CD56, where NK cells were defined as $CD3^-CD56^+$. The purity of healthy B cells was defined by their expression of CD20. The purity of the CLL cells was more challenging. Firstly, the relatively low expression of CD20 in CLL, and the variable expression of molecules such as CD23, made these molecules unsuitable targets for routine flow cytometry. Secondly, CD19 could not be used after positive isolation using CD19-microbeads due to either epitope occupation or CD19 internalisation after microbead binding. Instead CLL-cell purity was determined using antibodies to CD3 and CD5, where the CLL cells were defined as $CD3^-CD5^+$. The following purities were considered acceptable for downstream analysis:

| | |
|---|---|
| CLL B cells | > 95% |
| Healthy B cells | > 90% |
| Healthy/CLL $CD4^+$ T cells | > 95% |
| Healthy/CLL $CD8^+$ T cells | > 90% |
| Healthy/CLL NK cells | > 85% |

### *4.3.5 RNA extraction and quality control*

RNA was then immediately extracted from the isolated lymphocyte subsets using the RNAeasy Plus Mini Kit (Qiagen) as described in section 2.8. Quality control of the RNA samples was performed by spectrophotometric analysis (Nanodrop) to confirm the concentration and to detect contaminating proteins and other molecules, and RNA electrophoresis (Agilent) was used to determine the RNA integrity. The following values were considered acceptable for downstream analysis

| | |
|---|---|
| RNA concentration | > 25ng/µl |
| 260/230 | > 2.0 |
| 260/280 | > 2.0 |
| RNA Integrity Number | > 8.0 |

250ng RNA was used for the GeneChip arrays for CLL/healthy B cells and $CD4^+$ T cells; 125ng RNA was used for $CD8^+$ T cells and NK cells.

### *4.3.6 Affymetrix Human Genome U133Plus2.0 gene arrays*

RNA conversion to cDNA, *in vitro* transcription and subsequent hybridization to gene arrays using the GeneChip® 3' IVT Express Kit was performed according to the manufacturer's instructions (Affymetrix) as described in section 2.9. Briefly, RNA was reverse transcribed to synthesise first-strand cDNA using a T7 oligo(dT) primer to synthesize cDNA containing a T7 promoter sequence. The single-stranded cDNA was then converted into dsDNA using DNA polymerase and RNase H. The double-stranded cDNA was used as the template in an in vitro transcription (IVT) reaction catalysed by T7 polymerase and containing biotinylated CTP and UTP in addition to the 4 unmodified ribonucleoside triphosphates. The aRNA was then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the biotin-modified aRNA, before fragmented and prepared for hybridisation. The hybridisation to an Affymetrix HGU133Plus2.0 GeneChip and subsequent analysis using the GeneChip Fludics station 450 was performed by Tracy Chaplin in the Affymetrix core facility at Barts Cancer Institute. An initial quality control check was performed to exclude significant degradation in the sample aRNA. A number of control/housekeeping genes (e.g. actin, GAPDH) were included on the arrays with sequences covering the complete transcript. As RNA is usually degraded from the 5' to the 3' end loss of signal from probes for the transcripts towards the 5' end of the transcribed genes was a marker of any degradation of the RNA. 3':5' signal ratios of < 3 were used for downstream analysis.

### *4.3.7 Initial Biostatistical analysis*

To assess the impact of lenalidomide on gene expression profiling 8 paired (lenalidomide/DMSO control) samples were prepared for both healthy and CLL B cells, $CD4^+$ and $CD8^+$ T cells. 6 paired samples were used for healthy and CLL NK cells. The raw data was analysed by a dedicated biostatistician, Ajanthah Sangaralingham, at Barts Cancer Institute. Data was analysed within the R statistical environment using Bioconductor packages (http://www.bioconductor.org). Stringent quality control criteria were applied to the data, with probes that did not pass the quality control being removed. To detect differentially expressed genes between the subgroups, limma was used to fit a linear model to normalized expression data for each probe. False discovery rates (FDRs) were estimated using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). An FDR cutoff of 0.05 and an absolute fold change cutoffs of 2-3 were applied to filter the list of differentially expressed genes. Initial unsupervised

analysis showed clustering of the paired samples from each CLL patient/healthy control. Therefore gene lists were generated by supervised analysis; heatmaps were subsequently generated by using unsupervised hierarchical clustering using the gene lists. Pathways were analysed using Ingenuity Pathway Analysis software (www.ingenuity.com).

### *4.3.8 QRT-PCR*

The expression of twelve genes found to be differentially expressed after lenalidomide treatment was validated by QRT-PCR (**Table 4.1**). These were chosen to include those genes with the greatest fold-change in expression, and also to include gene of potential biological interest in the mechanism of action of lenalidomide. QRT-PCR was performed using the same RNA samples as were used for the gene microarrays. The method used was as described in section 2.10. 400 – 2000ng of RNA was used for cDNA synthesis, with 50ng of RNA being used for each Taqman® assay reaction.

**Table 4.1 Genes validated by QRT-PCR**

| Gene Name | Description | Taqman® Gene Expression Array |
|---|---|---|
| *CLDN12* | claudin 12 | Hs01082669_m1 |
| *F11R* | F11 receptor | Hs00170991_m1 |
| *CTNNA1* | catenin (cadherin-associated protein), alpha 1, 102kDa | Hs00944794_m1 |
| *TJP2* | tight junction protein 2 (zona occludens 2) | Hs00910543_m1 |
| *PPP2R3C* | protein phosphatase 2, regulatory subunit B", gamma | Hs00215595_m1 |
| *ARHGEF7* | Rho guanine nucleotide exchange factor (GEF) 7 | Hs00388776_m1 |
| *RHOC* | ras homolog gene family, member C | Hs00237129_m1 |
| *RND1* | Rho family GTPase 1 | Hs00205507_m1 |
| *ARL11* | ADP-ribosylation factor-like 11 | Hs00544739_m1 |
| *WASF1* | WAS protein family, member 1 | Hs01591751_m1 |
| *TPM2* | tropomyosin 2 (beta) | Hs00268540_m1 |
| *CYFIP1* | cytoplasmic FMR1 interacting protein 1 | Hs00383168_m1 |

### *4.3.9 Monoclonal antibodies*

The following directly conjugated monoclonal antibodies were used in this study: CD40-PECy7, CD58-FITC, CD80-PECy7, CD86-FITC, CD95-APC, CD279-APC and F11R-PE were all obtained from BD Biosciences; CD5-PerCPCy5.5, CD19-APCCy7, CD54-APC, CD59-FITC, CD134-FITC, CD154-PE and CD262-PE were all obtained from eBioscience; CD223-ATTO647 was obtained from Enzo Life Sciences.

### *4.3.10 Immunofluorescence staining and flow cytometric analysis*

Surface and intracellular staining for flow cytometric analysis was performed as described in section 2.7. Flow cytometry was performed on a BD Fortessa flow cytometer with subsequent analysis using FlowJo software (Tree Star). Analysis was performed on a minimum of 10,000 events after gating on live singlet cells. A fluorescent bead immunoassay for detection of levels of CCL4 in culture supernatants was performed using the Flowcytomix system from eBioscience according to the manufacturer's protocol: http://ebioscience.instant.at/bm_products/MAN/8420FF.pdf

## 4.4 Results

Gene expression profiling was performed after CLL or healthy PBMCs had been in cultured with lenalidomide or vehicle control for 24 – 48 hours. This *in vitro* model system had major advantages in terms of simplicity and experimental ease of use, but the period in culture meant that it was important to validate the system by comparing the results to data from *in vivo* analyses. This was done in several ways:

i) Comparison of the gene expression signature of untreated CLL cells in this dataset with previously published datasets in the literature.

ii) Comparison of the gene expression signature of untreated T cells from CLL patients in this dataset with previous work done by my group.

iii) Comparison of the effect of *in vitro* exposure to lenalidomide on the gene expression profiles of CLL cells in this dataset, with published data generated from *in vivo* exposure to lenalidomide in a clinical trial.

### *4.4.1 Comparison of the gene expression profiles of CLL cells with healthy B cells*

There have been several published analyses comparing the gene expression profiles of the malignant CLL B cells to healthy B cell subsets. The first two major analyses both concluded that CLL cells have a characteristic gene expression signature that was largely independent of their *IGVH* mutation status (Klein *et al.* 2001; Rosenwald *et al.* 2001). These observations are in contrast to the situation in diffuse large B-cell lymphoma, where the disease can be divided into two molecularly distinct forms based on gene expression profiling: “germinal centre B-like” and “activated B-like” (Alizadeh *et al.* 2000). The data from these first analyses was subsequently incorporated into a meta-analysis which also included a further cohort of samples where the gene expression profiling had been performed using nylon membrane arrays (Wang *et al.* 2004). This meta-analysis identified a number of genes that were consistently differentially expressed between CLL cells and healthy B cells. This dataset was used as a reference against which to compare differences in gene expression between CLL cells and healthy B cells in the control (i.e. lenalidomide untreated) condition.

**Table 4.2 Genes over-expressed in CLL cells**

| Symbol | Description | Abs. Fold Change |
|---|---|---|
| TNFRSF7 | Tumour necrosis factor receptor superfamily, member 7 (CD27) | 2.38 |
| MOX2 | Antigen identified by monoclonal antibody MRC OX-2 (CD200) | 3.14 |
| BCL2 | B-cell CLL/lymphoma 2 | 2.60 |
| TRA | T-cell receptor alpha locus (TRAC) | 6.28 |
| DSIPI | Delta sleep inducing peptide, immunoreactor (TSC22D3) | 1.97 |
| IL10RA | Interleukin 10 receptor, alpha | 2.22 |
| CDC25B | Cell division cycle 25B | 2.99 |
| FCGRT | Fc fragment of IgG, receptor, transporter, alpha | Not present |
| AIM2 | Absent in melanoma 2 | Not present |
| MXI1 | MAX interacting protein 1 | 2.22 |
| PTPN22 | Protein tyrosine phosphatase, non-receptor type 22 (lymphoid) | 2.68 |
| FGR | Gardner–Rasheed feline sarcoma viral (v-fgr) oncogene homolog | 4.69 |
| IL4R | IL4R Interleukin 4 receptor | 1.56 |
| PECAM1 | Platelet/endothelial cell adhesion molecule (CD31 antigen) | Not present |
| TTN | TTN Titin | 12.13 |
| PTPN12 | Protein tyrosine phosphatase, non-receptor type 12 | 4.32 |
| IL24 | IL24 Interleukin 24 | 1.66 |
| FCER2 | Fc fragment of IgE, low affinity II, receptor for (CD23A) | 5.31 |
| PPP2R5C | Protein phosphatase 2, regulatory subunit B (B56), gamma isoform | 2.55 |
| PNOC | Prepronociceptin | Not present |
| TNFSF1B | Tumour necrosis factor receptor superfamily, member 1B (LTA) | Downregulated |
| MHC2TA | MHC class II transactivator (CIITA) | Not present |
| PIM1 | pim-1 oncogene | 4.14 |
| CD5 | CD5 antigen (p56-62) | 11.71 |
| COPEB | Core promoter element binding protein (KLF6) | 1.54 |
| ALOX | Arachidonate 5-lipoxygenase | Not present |
| PLCL2 | Phospholipase C-like 2 | 2.57 |
| TOSO | Regulator of Fas-induced apoptosis (FAIM3) | 1.99 |
| C6orf32 | Chromosome 6 open reading frame 32 | Not present |
| NIFU | Nitrogen fixation cluster-like | Not present |
| ITGAX | ITGAX Integrin, alpha X (antigen CD11C, alpha polypeptide) | Not present |
| COL9A2 | Collagen, type IX, alpha 2 | 3.84 |
| CCND3 | Cyclin D3 | Not present |
| CG018 | CG018: NEDD4 binding protein 2-like 1 (N4BP2L1) | 1.92 |
| CD6 | CD6 antigen | 2.69 |
| FCGR2B | Fc fragment of IgG, low affinity IIb, receptor for (CD32) | Not present |
| PMAIP1 | Phorbol-12-myristate-13-acetate-induced protein 1 | 4.38 |
| LCK | Lymphocyte-specific protein tyrosine kinase | 2.95 |
| PPP3CC | Protein phosphatase 3, catalytic subunit, gamma isoform | Not present |

As shown in **table 4.2**, 67% of the genes that were over-expressed in the reference dataset were also found to be significantly unregulated when comparing the gene expression profiles of the *in vitro* cultured cells. These included genes encoding proteins of known importance in CLL, such as CD5, CD23, CD200, LCK, IL4R and BCL2. The expression of a select group of other genes that were highlighted in the first two analyses and of known importance in CLL, but were not identified in the meta-analysis, were also investigated (**Table 4.3)**. Again the data from the cultured cells was consistent with previous findings, with *FMOD* (Fibromodulin), *CTLA4* (Cytotoxic T-lymphocyte-associated protein 4), and *ROR1* (Receptor tyrosine kinase-like orphan receptor 1) being amongst the most upregulated genes in the new dataset.

**Table 4.3 Other over-expressed genes in CLL cells**

| Symbol | Description | Reference | Abs. Fold Change |
|---|---|---|---|
| FMOD | Fibromodulin | Klein *et al.* | 390.72 |
| WNT3 | Wingless-type MMTV integration site family 3 | Rosenwald *et al.* | 198.09 |
| CTLA4 | Cytotoxic T-lymphocyte-associated protein 4 | Rosenwald *et al.* | 144.01 |
| LEF1 | Lymphoid enhancer-binding factor 1 | Klein *et al.* | 30.91 |
| FILIP1L | Filamin A interacting protein 1-like | Klein *et al.* | 27.28 |
| RAPGEF3 | Rap guanine nucleotide exchange factor (GEF) 3 | Klein *et al.* | 23.92 |
| TGFBR3 | Transforming growth factor, beta receptor III | Klein *et al.* | 20.53 |
| ROR1 | Receptor tyrosine kinase-like orphan receptor 1 | Klein *et al.* | 17.51 |
| IGFBP4 | Insulin-like growth factor binding protein 4 | Klein *et al.* | 9.58 |
| ATXN1 | Ataxin 1 | Klein *et al.* | 9.38 |
| SELP | P-selectin (CD62) | Klein *et al.* | 8.34 |
| IGSF3 | Immunoglobulin superfamily, member 3 | Klein *et al.* | 6.11 |
| IL2RA | Interleukin 2 receptor, alpha | Rosenwald *et al.* | 3.16 |
| DGKA | Diacylglycerol kinase, alpha 80kDa | Rosenwald *et al.* | 3.07 |
| GCLC | Glutamate-cysteine ligase, catalytic subunit | Klein *et al.* | 2.71 |
| TBXA2R | Thromboxane A2 receptor | Klein *et al.* | 2.48 |
| KLF11 | Kruppel-like factor 11 (TIEG2) | Klein *et al.* | 2.16 |
| PRKCB | Protein kinase C, beta | Rosenwald *et al.* | 2.07 |
| MAP3K1 | Mitogen-activated protein KKK 5 (MEKK1) | Rosenwald *et al.* | 2.06 |
| SELPLG | Selectin P ligand (CD162) | Klein *et al.* | 1.80 |
| TXNIP | Thioredoxin interacting protein (HHCPA78) | Rosenwald *et al.* | 1.79 |

Absolute fold change (Abs. Fold Change) refers to the absolute fold change in gene expression observed from the new *in vitro* dataset, and does not refer to values published in the literature.

Similarly, 72% of the genes that were under-expressed in the reference dataset were also found to be significantly down-regulated in the new dataset. These included genes such as *CD83*, *MYC*, *CCL4*, *CD10*, *CD38* and *ADA* (**Table 4.4**). Once again, the expression of a select group of other genes that were highlighted in the first two analyses and of known importance in CLL, but were not identified in the meta-analysis, was also investigated (**Table 4.5**). The data from the cultured cells was again consistent with previous findings, with *CCL22*, *BID* (BH3 interacting domain death agonist), and *CDKN3* (Cyclin dependent kinase inhibitor 3) being amongst the most downregulated genes in the cultured dataset.

Therefore CLL cells retain remarkably consistent changes in the gene expression profile when compared with healthy B cells, even after 48 hours of culture. This also validates the gene expression datasets of the B cells cultured under control conditions. A list of the most highly ($\geq$ 8 fold absolute change in expression; $\log_2$ fold change $\geq$ 3) up-regulated and down-regulated genes in CLL cells compared with healthy $CD19^+$ B cells is included in **Appendix C**.

**Table 4.4 Genes underexpressed in CLL cells**

| Symbol | Description | Exp. Fold Change |
|---|---|---|
| SLU7 | Step II splicing factor SLU7 | Not present |
| CD83 | CD83 antigen | 3.32 |
| LMO2 | LIM domain only 2 (TTG2) | 22.47 |
| MYC | v-myc myelocytomatosis viral oncogene homolog (avian) | 7.62 |
| CYBB | Cytochrome b-245, beta polypeptide | 15.03 |
| GS3955 | GS3955 protein (TRIB2) | 23.43 |
| CD1C | CD1C antigen, c polypeptide | 96.34 |
| SORL1 | Homo sapiens cDNA: FLJ21930 fis, clone HEP04301 | 27.10 |
| NME1 | Non-metastatic cells 1, protein (NM23A) | 6.11 |
| IGL@ | IGL@ Immunoglobulin lambda locus | 19.97 |
| SLC2A5 | SLC2A5 Solute carrier family 2, member 5 | 15.45 |
| SLC2A3 | SLC2A3 Solute carrier family 2 , member 3 | 3.81 |
| SLAM | SLAM Signaling lymphocytic activation molecule (SLAM7) | 20.53 |
| SORL1 | SORL1 Sortilin-related receptor, L, A repeats-containing | 27.10 |
| CREM | cAMP responsive element modulator | 2.45 |
| RGS16 | Regulator of G-protein signaling 16 | Not present |
| KIAA0084 | KIAA0084 protein (RPTN1) | 3.25 |
| NP | Nucleoside phosphorylase (PNP) | 4.66 |
| BCL2A1 | BCL2-related protein A1 | 13.09 |
| CD22 | CD22 antigen | 3.27 |
| KIAA0870 | KIAA0870 protein (DENND3) | 25.11 |

| Symbol | Description | Exp. Fold Change |
|---|---|---|
| STK17A | Serine/threonine kinase 17a (DRAK1) | 1.56 |
| SATB1 | Special AT-rich sequence binding protein 1 | 3.16 |
| NFKB1 | Nuclear factor of kappa light gene enhancer in B-cells 1 | 1.80 |
| TNF | Tumour necrosis factor (TNF superfamily, member 2) | 3.73 |
| PRKACB | Protein kinase, cAMP-dependent, catalytic, beta | 16.80 |
| ID2 | Inhibitor of DNA binding 2 | 16.91 |
| PAICS | Phosphoribosylaminoimidazole carboxylase | 5.31 |
| IGHM | Immunoglobulin heavy constant mu | 61.39 |
| SCYA4 | Small inducible cytokine A4 (CCL4) | 10.93 |
| BUB1 | BUB1 budding uninhibited by benzimidazoles 1 homolog | 17.15 |
| STK15 | Serine/threonine kinase 15 | Not present |
| IFI30 | Interferon, gamma-inducible protein 30 | 4.66 |
| PSCDBP | Pleckstrin homology, Sec7 and coiled/coil domains, BP | Not present |
| ? | H.sapiens, clone MGC:3963 IMAGE:3621362 | Not present |
| MS4A1 | Membrane-spanning 4-domains, subfamily A, member 2 | 4.63 |
| BASP1 | Brain abundant, membrane attached signal protein 1 | 6.77 |
| BIN1 | Bridging integrator 1 | 1.96 |
| SSI1 | JAK binding protein (SOCS1) | 5.74 |
| CD38 | CD38 antigen (p45) | 9.51 |
| MADH7 | MAD, mothers against decapentaplegic homolog 7 (SMAD7) | 7.78 |
| LCP1 | Lymphocyte cytosolic protein 1 (L-plastin) | 3.07 |
| SCYB10 | Small inducible cytokine subfamily B, member 10 (CXCL10) | 23.92 |
| EIF4G1 | Eukaryotic translation initiation factor 4 gamma, 1 | Not present |
| GBP1 | Guanylate binding protein 1, interferon-inducible, 67 kDa | 51.63 |
| TRIO | Triple functional domain (PTPRF interacting) | 3.14 |
| ADA | Adenosine deaminase | 11.63 |
| GLRX | Glutaredoxin (thioltransferase) | 7.11 |
| HSPCA | Heat-shock 90 kDa protein 1, alpha (HSP90AA1) | Not present |
| CTPS | CTP synthase | 3.61 |
| EGR3 | Early growth response 3 | 8.69 |
| RGS1 | Regulator of G-protein signaling 1 | Not present |
| CSE1L | CSE1 chromosome segregation 1-like (yeast) | Not present |
| LBR | LBR Lamin B receptor | Not present |
| RBMS1 | RNA-binding motif, single-stranded interacting protein 1 | 2.20 |
| LDHA | Lactate dehydrogenase A | 3.14 |
| MME | Membrane metallo-endopeptidase (CD10) | Not present |
| TNFRSF5 | Tumour necrosis factor receptor superfamily, member 5 | Not present |
| ADSL | Adenylosuccinate lyase | 2.16 |
| JUP | Junction plakoglobin | 7.78 |
| NUP153 | Nucleoporin 153 kDa | 2.10 |
| IL7R | Interleukin 7 receptor | 5.78 |
| NOL1 | Nucleolar protein 1 (NSUN5) | Not present |
| GPRK5 | G-protein-coupled receptor kinase 5 | Not present |
| CDC20 | CDC20 cell-division cycle 20 homolog | 18.25 |
| BLMH | Bleomycin hydrolase | 2.51 |
| SKIL | SKI-like | 1.87 |
| BCL2L1 | BCL2-like 1 | 2.19 |

| Symbol | Description | Exp. Fold Change |
|---|---|---|
| CR2 | Complement component (3d/Epstein–Barr virus) receptor 2 | Not present |
| IFITM1 | Interferon-induced transmembrane protein 1 (9–27) | 3.34 |
| DUSP2 | Dual specificity phosphatase 2 | Not present |
| HIF1A | Hypoxia-inducible factor 1, alpha subunit | Not present |
| PEA15 | Phosphoprotein enriched in astrocytes 15 | 2.30 |
| MAD2L1 | MAD2 mitotic arrest deficient-like 1 (yeast) | 4.23 |
| DAPK1 | Death-associated protein kinase 1 | 11.39 |
| VDR | Vitamin D (1,25-dihydroxyvitamin D3) receptor | Not present |
| CRIP2 | Cysteine-rich protein 2 | Not present |
| BLR1 | Burkitt lymphoma receptor 1, GTP-binding protein | Not present |
| CDC2 | Cell-division cycle 2, G1 to S and G2 to M | 30.48 |
| PPP1R16B | Protein phosphatase 1, regulatory (inhibitor) subunit 16B | Not present |
| CENPF | Centromere protein F (350/400 kDa, mitosin) | 4.47 |
| NEK2 | NIMA (never in mitosis gene a)-related kinase 2 | 6.59 |
| EGFL5 | EGF-like-domain, multiple 5 | Not present |
| PLCG2 | Phospholipase C, gamma 2 (phosphatidylinositol-specific) | Not present |
| RGS13 | Regulator of G-protein signaling 13 | Upregulated |

## Table 4.5 Other genes underexpressed in CLL cells

| Symbol | Description | Reference | Exp. FC |
|---|---|---|---|
| CCL22 | Macrophage derived chemokine | Klein *et al.* | 178.53 |
| BID | BH3 interacting domain death agonist | Klein *et al.* | 17.88 |
| CIP2 PTP | Cyclin-dependent kinase inhibitor 3 (CDKN3) | Klein *et al.* | 16.34 |
| EC-2 | Ubiquitin-conjugating enzyme E2C (UBE2C) | Klein *et al.* | 12.91 |
| CCR6 | Chemokine (C-C motif) receptor 6 | Klein *et al.* | 9.32 |
| CD18 | Integrin, beta 2 (ITGB2) | Klein *et al.* | 8.28 |
| P1CDC21 | Minichr. maintenance complex component 4 (MCM4) | Klein *et al.* | 6.73 |
| Cyclin B | Cyclin B1 (CCNB1) | Klein *et al.* | 5.90 |
| fls353 | TPX2, microtubule-associated, homolog (TPX2) | Klein *et al.* | 4.82 |
| Zyxin | Zyxin (ZYX) | Klein *et al.* | 4.00 |
| ICAM1 | Intercellular adhesion molecule 1 | Klein *et al.* | 4.00 |
| LFA-1α | Integrin, alpha L (antigen CD11A, ITGAL) | Klein *et al.* | 3.46 |
| DRAK2 | Serine/threonine kinase 17b (STK17B) | Klein *et al.* | 2.95 |
| DP1 E2F | Transcription factor Dp-1 (TFDP1) | Klein *et al.* | 2.68 |
| TNFSF11 | TNF (ligand) superfamily, 11 (Osteoprotein ligand) | Klein *et al.* | 2.27 |
| Fascin | FSCN1 | Klein *et al.* | 1.65 |

### *4.4.2 Comparison of the gene expression profiles of $CD3^+CD4^+$ T cells from CLL patients with healthy $CD3^+CD4^+$ T cells*

My group has previously compared the gene expression profiles of peripheral blood T cells from previously untreated patients with CLL with those from age-matched healthy donors (Gorgun *et al.* 2005). Despite the cells not being part of the malignant clone, analysis revealed significant alterations in gene expression. Furthermore, these changes could be induced in healthy T cells by co-culturing them with the malignant CLL cells. In $CD4^+$ T cells, the majority of the differentially expressed genes were involved in cell differentiation and proliferation, survival, cytoskeleton formation, and vesicle trafficking. In particular, there was decreased expression in a number of genes in the Ras-dependent JNK and p38 MAPK pathways, including *MINK* (*MAP4K6*), *GDI1*, and *NFRKB*. Genes involved in regulating cell growth (e.g. *PIK3CB*) and in cytoskeleton formation and vesicle trafficking were also prominently dysregulated (e.g. *AAK1*, *AP3M2*, *SPTBN1*, *ARPC1*, and *ADIR*).

As a part of the validation of the gene expression profiling performed here, the genes that were differentially expressed between healthy and CLL $CD4^+$ T cells in the control condition were compared with the published dataset (Gorgun *et al.* 2005). The alterations in gene expression were modest with only 8 probes that were significantly up-regulated and 14 probes that were significantly down-regulated (> 1.5 absolute fold change; $P < 0.05$). None of the same genes that had been identified as dysregulated n the published dataset recurred in the new dataset. Despite this, dysregulation of the JNK and p38 MAPK pathways was still apparent, with decreased expression of both *ATF2* (activating transcription factor 2), and *RPS6KA5* (ribosomal protein S6 kinase, polypeptide 5). Indeed this pathway was highlighted in an ingenuity analysis of this dataset (**Figure 4.1**). The complete list of dysregulated probes is in **Appendix D**.

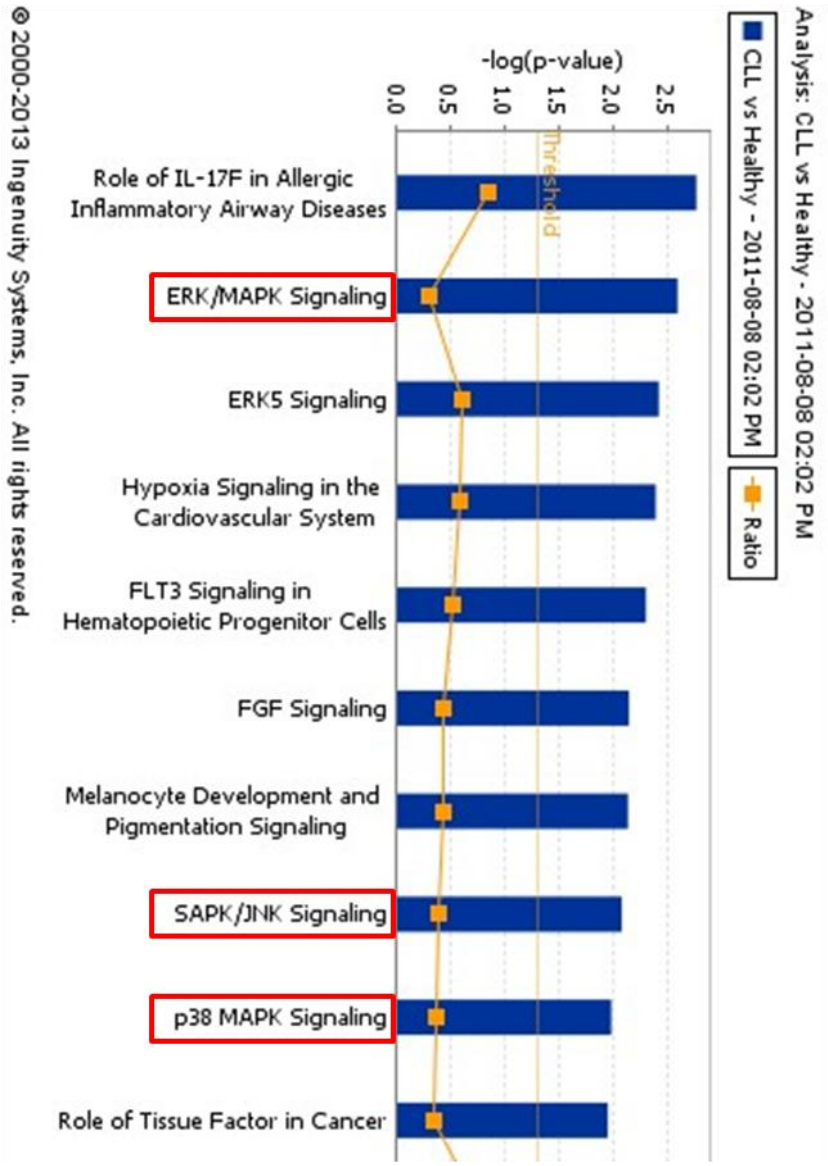



**Figure 4.1 Ingenuity pathways analysis of dysregulated genes in CLL $CD4^+$ T cells**

### *4.4.3 Comparison of the gene expression profiles of $CD3^+CD8^+$ T cells from CLL patients with healthy $CD3^+CD8^+$ T cells*

Previous work by my group had identified a greater degree of dysregulation in the gene expression profiles of circulating $CD8^+$ T cells (Gorgun *et al.* 2005). These observations highlighted defects in several cellular pathways, including cytoskeleton formation, intracellular transportation, vesicle trafficking, and cytotoxicity. In particularly there was dysregulation of a number of cytoskeletal genes including down-regulation of *ARAP3*; *myosin IXB*; *AP3M2*; *VAMP2*; *GPR57*; and *AKAP9*, and up-regulation of *CDC42*; *PIK4CB*; *RAB35*; *FLNA*; *FMNL*; *ARPC1B*; *SPEC1*; *NCK2*; *RAB22A*; *RALGDS*; and *RASGRP2*.

As above, these previously published observations were compared with the genes differentially expressed in CLL $CD8^+$ T cells in the new dataset. Of the 36 genes highlighted as being dysregulated in the original publication, only 2 (6%) recurred in the new dataset. These were *SPTB* (sprectrin beta) and *RALGDS* (ral guanine nucleotide dissociation stimulator): both of which are implicated in cytoskeletal regulation. However there was down-regulation of several other important cytoskeletal genes in CLL $CD8^+$ T cells including *WASF1*, *RAB13*, *RHOH*, *ACTN1* and *CAMSAP1L1*, with "leucocyte extravasation signalling" being highlighted by Ingenuity pathways analysis as the most the dysregulated pathway (**Figure 4.2)**. A list of the most dysregulated genes ($\geq$ 3 fold change dysregulated) is given in **Appendix E**.

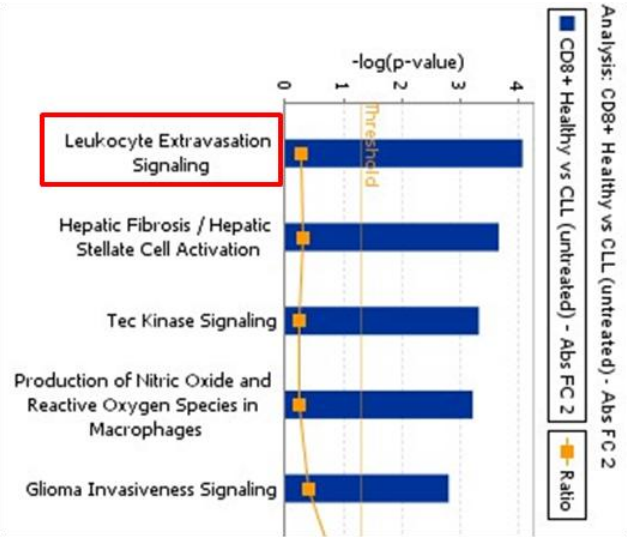



**Figure 4.2 Ingenuity pathways analysis of dysregulated genes in CLL $CD8^+$ T cells**

### *4.4.4 $CD3^+CD8^+$ T cells from CLL patients have altered expression of genes implicated in T-cell exhaustion*

Given the findings described in chapter 3, the new dataset was also examined for alterations in gene expression consistent with T-cell exhaustion. Importantly, there was dysregulation of several genes implicated in this process, including up-regulation of *LAG3*, *KLRG1*, and *PRDM1* (BLIMP1), and down-regulation of *IL7R* (**Table 4.6**). This confirms that the observed changes in protein expression and cytokine production are also present at the mRNA level.

**Table 4.6 The expression of "Exhaustion" genes in $CD8^+$ T cells from CLL patients**

| **Upregulated** | | | |
|---|---|---|---|
| **Symbol** | **Description** | **Absolute Fold change** | **Adjusted P value** |
| IFNG | Interferon, gamma | 5.8 | 0.004 |
| LAG3 | Lymphocyte-activation gene 3 | 2.7 | 0.009 |
| KLRG1 | Killer cell lectin-like receptor subfamily G, member 1 | 2.6 | 0.001 |
| FASLG | Fas ligand (TNF superfamily, member 6) | 2.4 | 0.026 |
| PRDM1 | PR domain containing 1, with ZNF domain (BLIMP1) | 2.1 | 0.014 |
| TBX21 | T-box 21 (TBET) | 2.0 | 0.047 |
| EOMES | Eomesodermin | 1.9 | 0.014 |
| **Downregulated** | | | |
| **Symbol** | **Description** | **Absolute Fold change** | **Adjusted P value** |
| IL7R | Interleukin 7 receptor | 1.5 | 0.040 |

### *4.4.5 Comparison of the gene expression profiles of CD3$^-$CD56$^+$ NK cells from CLL patients with healthy CD3$^-$CD56$^+$ NK cells*

While my group has compared the gene expression profiles of T cells from CLL patients with healthy controls, a comparison of the transcriptome of *NK cells* from CLL patients and controls has not previously been investigated. In light of this, a comparison was made of the gene expression profiles of NK cells from the control (i.e. lenalidomide untreated) condition, after 24 hours in culture. 18 probes were showed a >2-fold increase in expression, and 117 probes showed a >2-fold decrease in expression after filtering for probes with an adjusted p-value < 0.05.

A particularly striking feature of the genes which were down-regulated in CLL NK cells was that they were enriched for interferon-inducible genes. 52 out of the 117 probes (44.4%) that were significantly down-regulated > 2-fold were for interferon inducible genes, including *STAT1*, *SOCS1*, and *IRF9* (**Figure 4.3/Table 4.7**). These were regulated by type 1 interferons (e.g. IFNα and IFNβ) and type 2 interferons (e.g. IFNγ), with the majority of these genes being inducible by both types. Many of these genes have been implicated in host immunity to viral infections, and so it is possible that decreased NK-cell responsiveness to interferon contributes to the increased susceptibility of CLL patients to viruses. There was also altered expression of the same pathways that were dysregulated in CLL T cells. *JUN* was significantly down-regulated in NK cells from CLL patients, which would correspond to the down-regulation in JNK-signalling in CD4$^+$ T cells. Dysregulation of a number of cytoskeleton genes was also observed including *RAB3GAP1*, *RAB38*, and *EPHA1*. These changes were not due to differences in the relative frequencies of CD56$^{DIM}$ and CD56$^{BRIGHT}$ NK cells. A list of the most dysregulated genes (≥ 3 fold change dysregulated) is given in **Appendix F**.

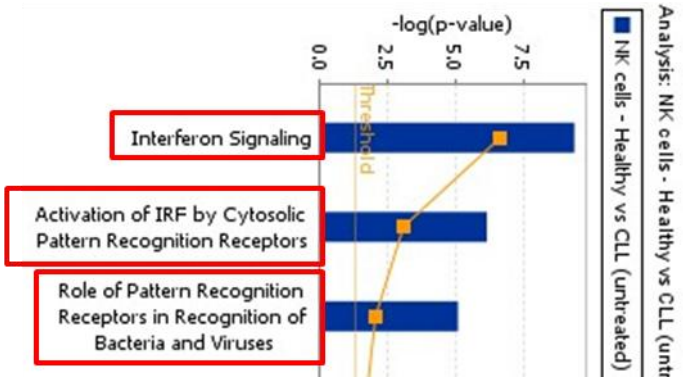



**Figure 4.3 Ingenuity pathways analysis of dysregulated genes in CLL NK cells**

**Table 4.7 Down-regulation of IFN-inducible genes in CLL $CD3^{-}CD56^{+}$ NK cells**

| Symbol | Description | $Log_2$ Fold change | Adjusted P value |
|---|---|---|---|
| IFI44L | Interferon-induced protein 44-like | 6.529 | 6.0E-05 |
| IFIT1 | Interferon-induced protein with tetratricopeptide repeats 1 | 4.212 | 4.7E-05 |
| RSAD2 | Radical S-adenosyl methionine domain containing 2 | 4.018 | 6.0E-05 |
| IFI44 | Interferon-induced protein 44 | 3.237 | 3.5E-04 |
| OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa | 3.201 | 4.2E-04 |
| OAS1 | 2'-5'-oligoadenylate synthetase 1, 40/46kDa | 3.108 | 1.1E-03 |
| MX1 | Myxovirus resistance 1, interferon-inducible protein p78 | 3.061 | 3.6E-03 |
| IFIT3 | Interferon-induced protein with tetratricopeptide repeats 3 | 2.996 | 4.8E-04 |
| IFI6 | Interferon, alpha-inducible protein 6 | 2.540 | 7.1E-04 |
| EIF2AK2 | Eukaryotic translation initiation factor 2-alpha kinase 2 | 2.354 | 1.4E-03 |
| JUN | Jun proto-oncogene | 2.119 | 7.4E-05 |
| SOCS1 | Suppressor of cytokine signaling 1 | 2.086 | 1.2E-03 |
| USP18 | Ubiquitin specific peptidase 18 | 2.048 | 2.1E-03 |
| EPSTI1 | Epithelial stromal interaction 1 (breast) | 1.978 | 4.7E-05 |
| CCRL2 | Chemokine (C-C motif) receptor-like 2 | 1.880 | 3.0E-02 |
| MX2 | Myxovirus resistance 2 | 1.873 | 1.2E-02 |
| OASL | 2'-5'-oligoadenylate synthetase-like | 1.870 | 4.8E-04 |
| PMAIP1 | Phorbol-12-myristate-13-acetate-induced protein 1 | 1.858 | 1.8E-02 |
| ISG15 | ISG15 ubiquitin-like modifier | 1.724 | 1.9E-02 |
| HERC6 | HECT & RLD domain containing E3 ubiquitin protein ligase 6 | 1.718 | 5.2E-04 |
| IRF7 | Interferon regulatory factor 7 | 1.556 | 7.9E-04 |
| IFIT2 | Interferon-induced protein with tetratricopeptide repeats 2 | 1.542 | 2.8E-02 |
| HERC5 | HECT & RLD domain containing E3 ubiquitin protein ligase 5 | 1.514 | 4.6E-03 |
| XAF1 | XIAP associated factor 1 | 1.511 | 7.0E-03 |
| BTG1 | B-cell translocation gene 1, anti-proliferative | 1.387 | 1.7E-03 |
| STAT1 | Signal transducer and activator of transcription 1, 91kDa | 1.363 | 2.2E-03 |
| DUSP10 | Dual specificity phosphatase 10 | 1.335 | 3.9E-03 |
| PLSCR1 | Phospholipid scramblase 1 | 1.203 | 2.4E-03 |
| CXCR4 | Chemokine (C-X-C motif) receptor 4 | 1.132 | 1.0E-02 |
| OAS2 | 2'-5'-oligoadenylate synthetase 2, 69/71kDa | 1.091 | 1.0E-02 |
| ETS2 | v-ets erythroblastosis virus E26 oncogene homolog 2 | 1.091 | 1.8E-03 |
| IFIT5 | Interferon-induced protein with tetratricopeptide repeats 5 | 1.091 | 1.4E-03 |
| IRF9 | Interferon regulatory factor 9 | 1.091 | 3.1E-03 |

### *4.4.6 The impact of lenalidomide on the gene expression profiles of CLL cells*

In order to investigate the effect of lenalidomide on the gene expression profiles of CLL cells, PBMCs from CLL patients were cultured in the presence of 1μM lenalidomide or vehicle control for 48 hours, followed by RNA extraction and gene expression profiling. Lenalidomide was primarily an activator of gene expression with 357 probes being significantly (adjusted P-value < 0.05) up-regulated with >2-fold increase in expression and 173 probes being significantly down-regulated with >2-fold decrease in expression. Lenalidomide had significant effects on genes implicated in a variety of different pathways (**Figure 4.4**):

- TNF family: *TNF*, *TNFRSF4* (OX40), *TNFSF4* (OX40L), *TNFRSF13B* (TACI), *TNFSF13* (APRIL), *TNFRSF10B* (DR5), *TNFRSF10D* (DCR2)
- Adhesion molecules: *ICAM1*, *CLDN12*, *JAM2*, *CD58* (LFA1), *ITGAX* (CD11c)
- Cytoskeletal pathways: *COL9A*, *TPM2*, *RND1*, *RIN2*, *SNX8*, *MAP2*, *WASF1*, *RAPGEF5*, *RAB13*, *EMILIN2*, *FMNL3*, *FILIP1L*, *RAB20*, *ARHGAP31*, *RASGRP2*, *COL18A1*, *COL9A3*, *ANK1*, *FLNA*, *RAB37*, *ARHGEF10L*
- Cell cycle: *CDKN1C*, *CDKN1A*, *CCND2*, *CCND1*
- Apoptosis pathways: *BID*, *BIK*, *FAS*, *HRK*
- Chemokines, chemokines receptors and B cell activation markers: *CCL4*, *CCL2*, *CCR2*, *CCR6*, *LAG3*, *CD70*, *CD83*, *CD274*, *CD72*, *CD200*
- Genes associated with chromosome 5q: *SPARC*, *PPP2R3C*

Overall, lenalidomide treatment led to CLL-cell activation in this assay, with increased expression of several activation markers and chemokines/chemokine-receptors, increased adhesion molecule expression, and up-regulation of pro-proliferative genes. There was up-regulation of several genes involved in cytoskeletal pathways, which is also likely to reflect CLL cell activation, and is consistent with my group's previous observations regarding improved CLL-cell antigen presenting function and immunological synapse formation after lenalidomide treatment (Ramsay *et al.* 2008). A list of the genes that were most affected by lenalidomide treatment ($\geq$ 3 fold change) is given in **Appendix G**.

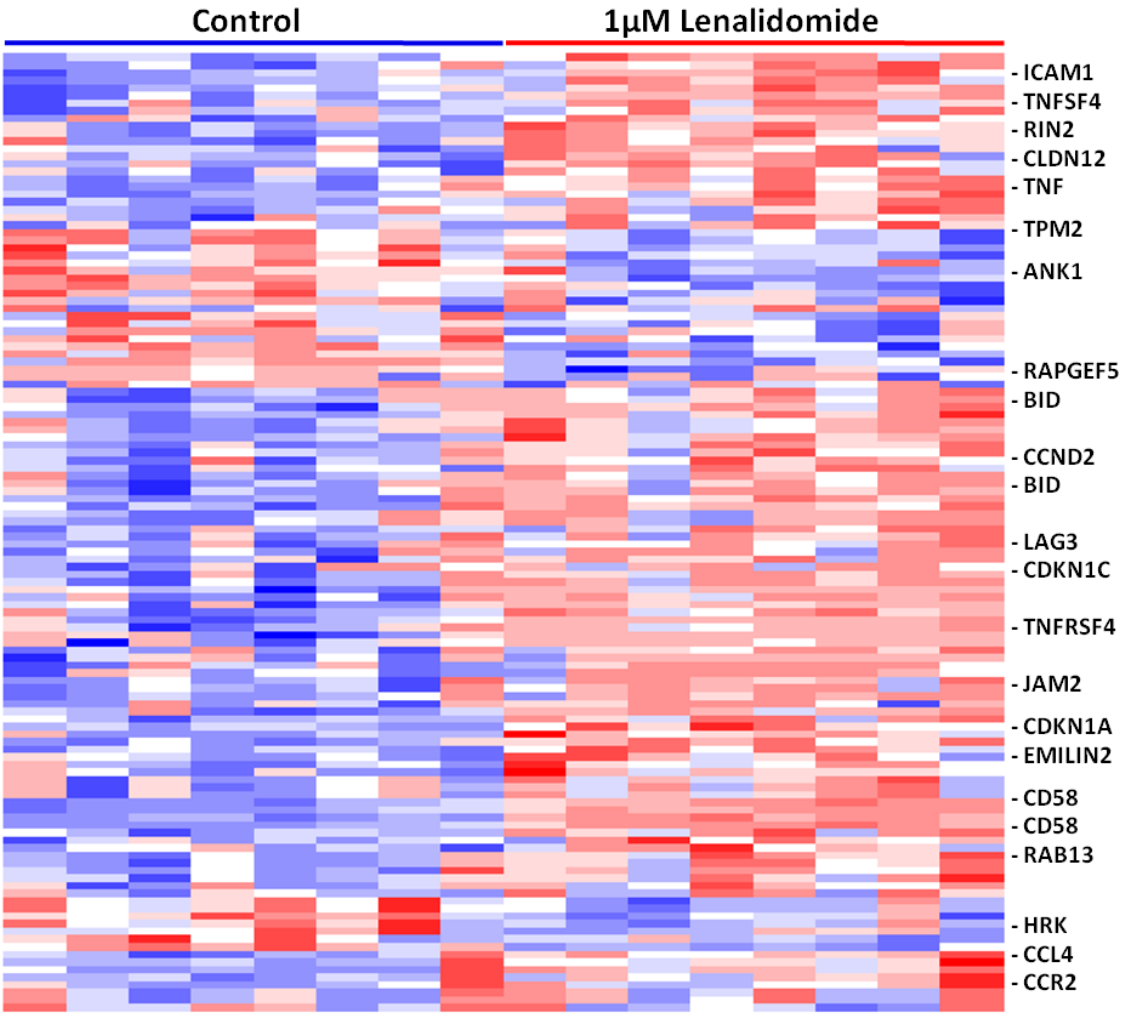



**Figure 4.4 Heatmap showing genes with > 3-fold change in expression after lenalidomide treatment in CLL cells**

Red = upregulated; Blue = downregulated

### *4.4.7 Comparison of the in vitro findings with in vivo results from a clinical study*

Lenalidomide has been shown to have efficacy in previously untreated CLL patients, with overall response rates of 72% in a recent clinical trial (Chen *et al.* 2012). Correlative studies were performed as part of this trial to assess the impact of lenalidomide treatment on the gene expression profiles of PBMCs enriched for CLL cells (purity > 85%) (Chen *et al.* 2010). Gene expression profiling was performed on enriched PBMCs taken on day 1 (pre-dosing) and on day 8 from 24 patients. Affymetrix Genechip U133 Plus2.0 microarrays were used for this analysis, the same arrays as used for the *in vitro* studies described in this thesis. A lenalidomide-induced signature representing 42 differentially expressed gene with at least a 2-fold difference in expressed was identified, with 26 genes up-regulated, and 16 genes down-regulated. The altered genes were enriched for immune-associated genes including *TNF* and *LAG3*, and IFN-induced genes such as *OAS3* (2,5-oligoadenylate synthetase). Notably, the most up-regulated gene in this analysis was *TPM2* (tropomyosin 2), a cytoskeletal regulatory protein.

A part of the validation of the *in vitro* dataset, this data and the lenalidomide-induced gene expression signature from the clinical trial were directly compared (**Figure 4.5**). 27 of the 46 (59%) differentially expressed probes detected in the clinical trial also showed statistically significant changes in expression in the *in vitro* dataset. This included 21 out of the 29 (72%) upregulated probes, and 6 out of the 17 (35%) downregulated genes. These included the genes highlighted in the clinical trial report: *TNF*, *LAG3*, *OAS3* and *TPM2*. This data provides further validation of the *in vitro* PBMC model system.

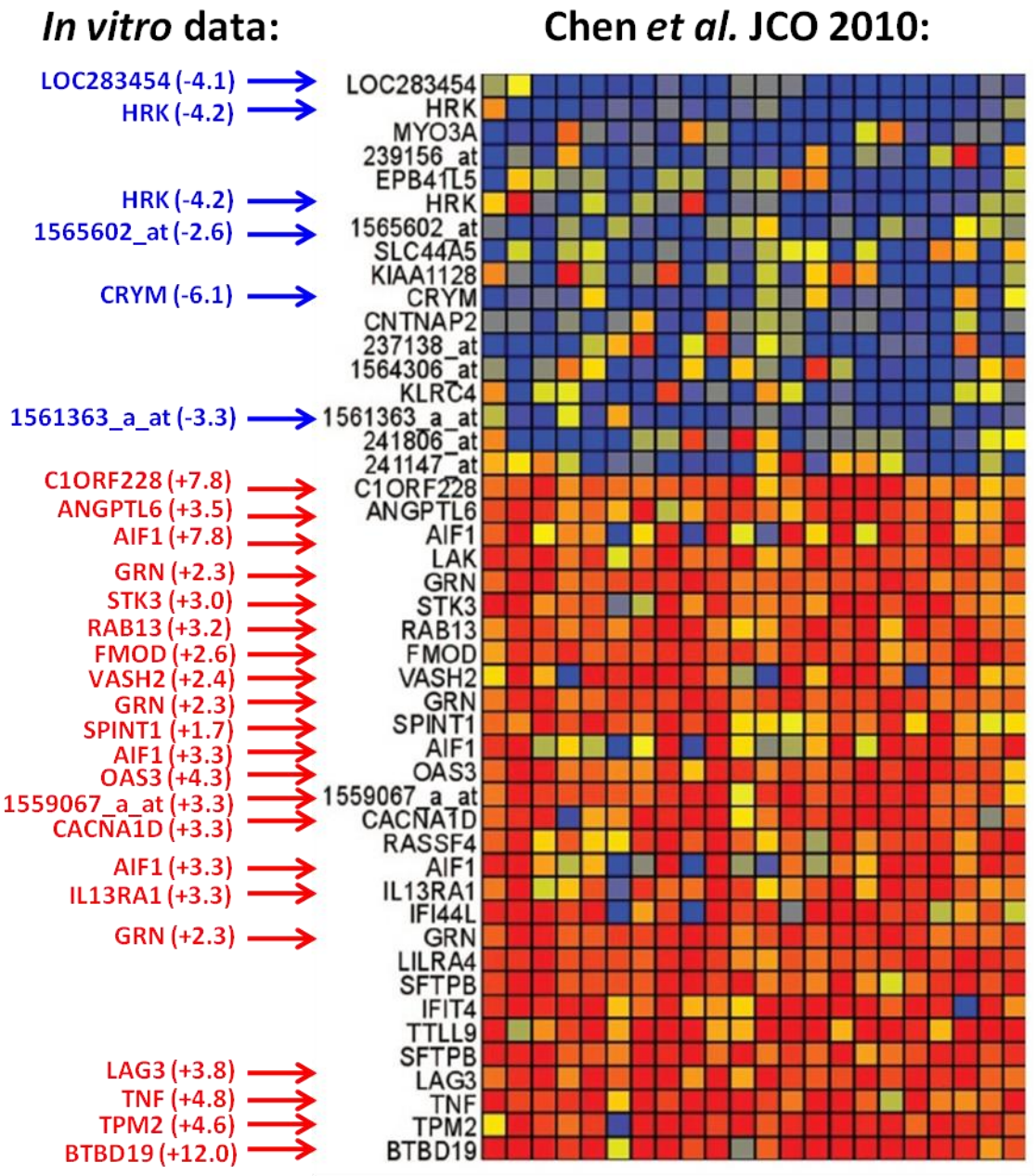



**Figure 4.5 Comparison of the *in vitro* dataset with published data from a clinical trial (Chen *et al.* 2010)**

Published data from a clinical trial was compared with the *in vitro* dataset in order to assess which genes were dysregulated in both analyses. The overlapping genes are highlighted in colour in the left hand column (blue = downregulated; red = upregulated). The fold change in expression in the *in vitro* dataset was highlighted in brackets.

### *4.4.8 The impact of lenalidomide on the gene expression profiles of healthy B cells*

The effect of lenalidomide on the transcriptome of healthy B cells was also analysed. Interestingly, lenalidomide had less of an effect here, with only 130 probes being significantly up-regulated with >2-fold increase in expression and 107 probes being significantly down-regulated with >2-fold decrease in expression. When analysing the most highly altered probes (>3-fold changes), only 30 probes were significantly up-regulated in healthy B cells, compared to 98 in CLL cells, and only 17 probes were significantly down-regulated, compared to 29 in CLL cells.

Lenalidomide had effects on genes in some of the same pathways as observed in CLL cells (**Figure 4.6**):

- TNF family: *TNFSF4* (OX40L), *TNFRSF11B*, *TNFSF11*, *TNFRSF10D* (DCR2)
- Adhesion molecules: *CLDN12*, *JAM2*
- Cytoskeletal pathways: *RND1*, *WASF1*, *FMNL3*, *RAB37*, *ARHGEF5*, *RHOQ*, *MYO1B*, *ARHGAP20*, *CAMSAP2*, *COL1A1*
- Genes associated with chromosome 5q: *SPARC*

Interestingly, there were no significant changes in the expression of genes implicated in the cell cycle, apoptosis, and B-cell activation/chemokine signalling pathways that were observed in CLL cells with lenalidomide treatment. This would suggest that lenalidomide activates the cytoskeleton and up-regulates the expression of TNF family members and adhesion molecules directly, but has a co-stimulatory effect with regard to B-cell activation and proliferation, augmenting events induced by BCR-signalling. This would also suggest that there is ongoing tonic BCR-signalling in CLL cells, which is not present in healthy B cells in this PBMC culture system. A list of the genes that were most affected by lenalidomide treatment ($\geq$ 3 fold change) is given in **Appendix H**.

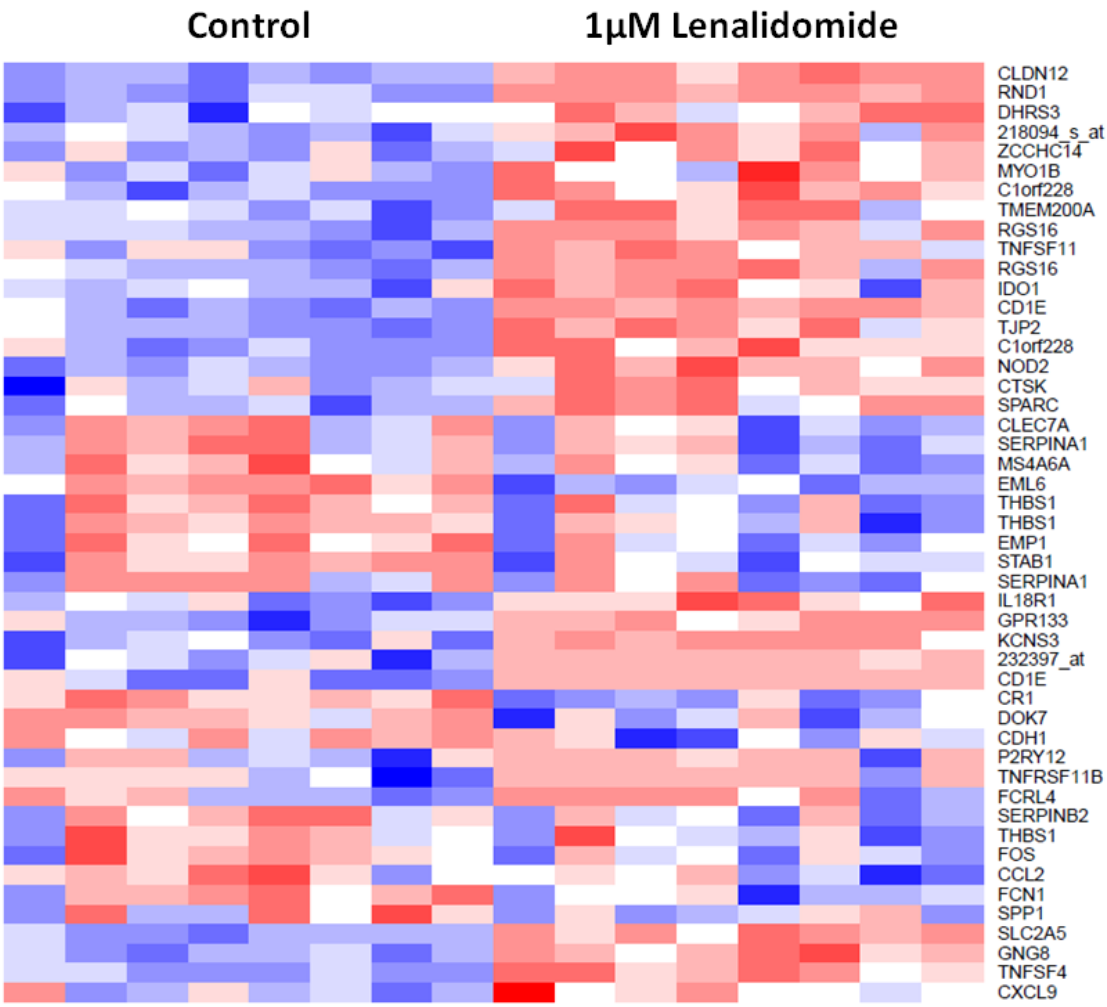



**Figure 4.6 Heatmap showing genes with > 3-fold change in expression after lenalidomide treatment in healthy CD19$^{+}$ B cells**

Red = upregulated; Blue = downregulated

### *4.4.9 Validation of Affymetrix array observations: upregulation of surface proteins on CLL cells with lenalidomide treatment*

The gene expression profiling data from the Affymetrix HGU133Plus2.0 microarrays was validated at the protein level. As several of the up-regulated genes coded for B-cell activation markers that were surface molecules, the expression of these proteins was determined by flow cytometry. PBMCs from an independent cohort of 40 CLL patients were cultured *in vitro* for 48 hours with 1μM lenalidomide or vehicle control, using the same culture conditions as used for gene expression profiling. A panel of 12 surface markers were selected including:

- CD134 (TNFRSF4; OX40), CD54 (ICAM1), CD59, CD223 (LAG3), CD279 (PD1), CD58 (LFA3) , CD95 (FAS), and CD262 (TNFRSF10B; DR5) on the basis of being significantly up-regulated ≥ 2-fold in the array dataset
- CD40, CD80, CD86, and CD154 (CD40L), on the basis of having been previously described as up-regulated by lenalidomide treatment (Andritsos *et al.* 2008; Aue *et al.* 2009; Lapalombella *et al.* 2010)

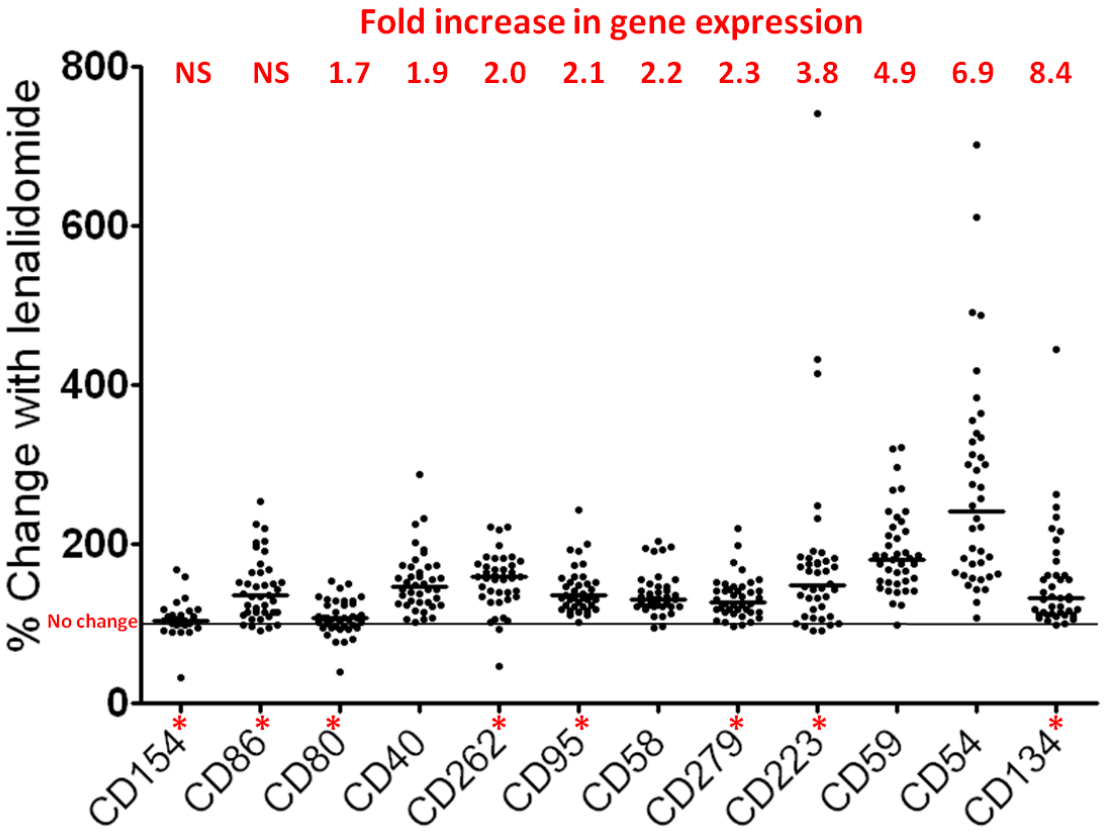



**Figure 4.7 Validation of the changes in expression of genes encoding CLL cell surface proteins**

The % change with lenalidomide treatment of surface protein expression as measured by MFI is shown normalised to the untreated condition. The fold-changes from the array dataset are highlighted in red.

**Figure 4.7** summarises the findings. All of the data points shown were generated by normalising the median fluorescence intensity (MFI) in the treated condition to the paired control sample. This approach was most appropriate for proteins that were *constitutively* expressed by CLL cells, such as CD40, CD58, CD59, and CD54. However proteins marked by a red asterisk are *inducibly* expressed by CLL cells. In particular, CD223 and CD134 were not expressed on CLL cells in the control condition, but were significantly up-regulated by a subset of CLL cells with lenalidomide treatment (**Figure 4.8**). This change is best described by an increase in the percentage of cells positive for a given antigen, but MFI was used here to allow comparison between all of the surface markers.

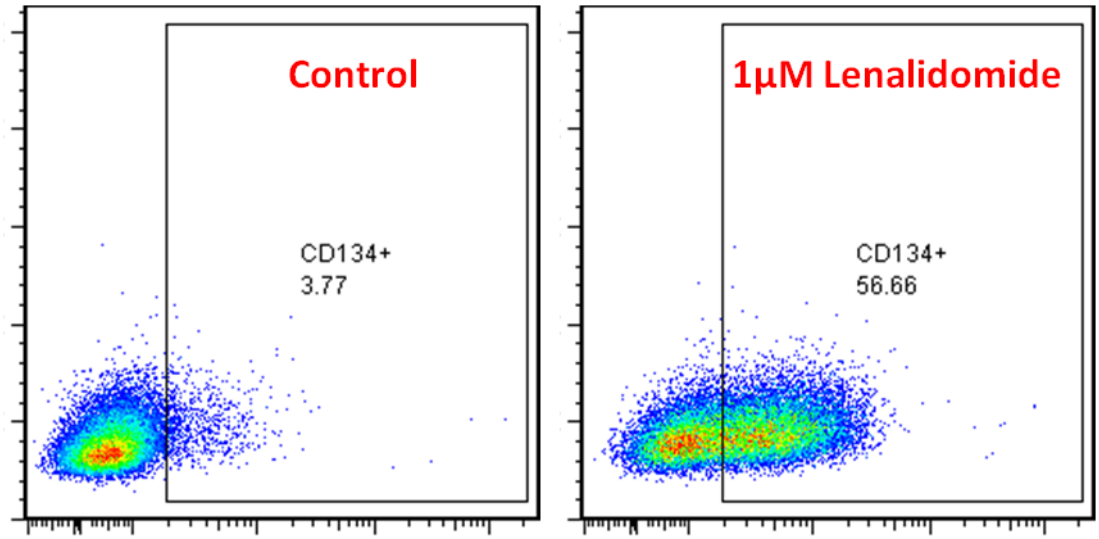



**Figure 4.8 Increased expression of CD134 (OX40) on a subset of CLL cells with lenalidomide treatment**

Flow cytometric plot showing an large expansion of $CD134^+$ CLL cells with lenalidomide treatment.

Taking this into account, there was a qualitative correlation between the fold-change in gene expression in the array data, and the degree of up-regulation of the surface protein with lenalidomide treatment. CD223, CD59, CD54 and CD134 showed the greatest changes in mRNA levels with lenalidomide treatment in the array dataset, and also showed the biggest changes in surface molecule expression by flow cytometry.

### *4.4.10 Validation of Affymetrix array observations: Lenalidomide treatment increases production of CCL4 by CLL cells.*

The chemokine gene *CCL4* was one of the genes that was most up-regulated by lenalidomide treatment, with >7-fold increase in mRNA expression. The chemokines CCL3 and CCL4 have implicated in the pathogenesis of CLL in several recent publications. Co-culture of CLL cells with nurse-like cells (NLCs) induces high-level expression of these chemokines by the tumour cells, with the degree of induction correlating with ZAP70 expression. NLC-induced production of CCL3/CCL4 could be inhibited by a Syk inhibitor; conversely BCR-triggering was noted to cause robust CCL3/CCL4 protein secretion by CLL cells (Burger *et al.* 2009). There is also evidence to suggest that serum levels of these chemokines have prognostic significance in CLL and DLBCL (Sivina *et al.* 2011; Takahashi *et al.* 2012). Interestingly, serum CCL3 and CCL4 levels were also shown to correlate with response in elderly patients treated with lenalidomide in a clinical trial, with responders having lower levels than non-responders (Badoux *et al.* 2011). In light of this the ability of lenalidomide to induce CLL-cell secretion of CCL4 was assessed. Lenalidomide treatment induced a dose- and time-dependent increase in CCL4 production by CLL cells (**Figure 4.9**).

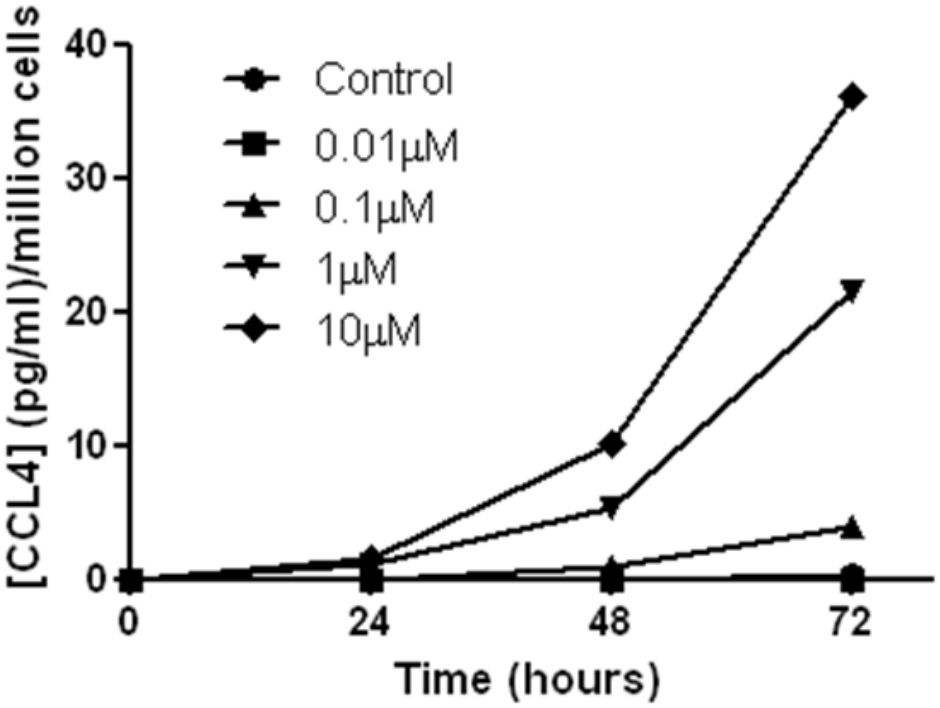



**Figure 4.9 Lenalidomide induces the secretion of CCL4 by CLL cells**

The ability of lenalidomide to induce CLL-cell secretion of CCL4 was assessed. Purified CLL cells were cultured in the presence of various concentrations of lenalidomide for up to 72 hours. Culture supernatant CCL4 levels were measured using a cytokine bead assay.

### *4.4.11 The impact of lenalidomide on the gene expression profiles of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells from CLL patients*

The impact of lenalidomide on the gene expression profiles of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells from CLL patients was also investigated. As before PBMCs from CLL patients were cultured in the presence of 1μM lenalidomide or vehicle control for 48 hours, followed by RNA extraction and gene expression profiling using the Affymetrix U133 Plus 2.0 arrays. Once again, lenalidomide was primarily an activator of gene expression with 231/188 probes being significantly up-regulated with >2-fold increase in expression in $CD3^+CD4^+$/$CD3^+CD8^+$ T cells and 47/65 probes being significantly down-regulated with >2-fold decrease in expression in $CD3^+CD4^+$/$CD3^+CD8^+$ T cells respectively.

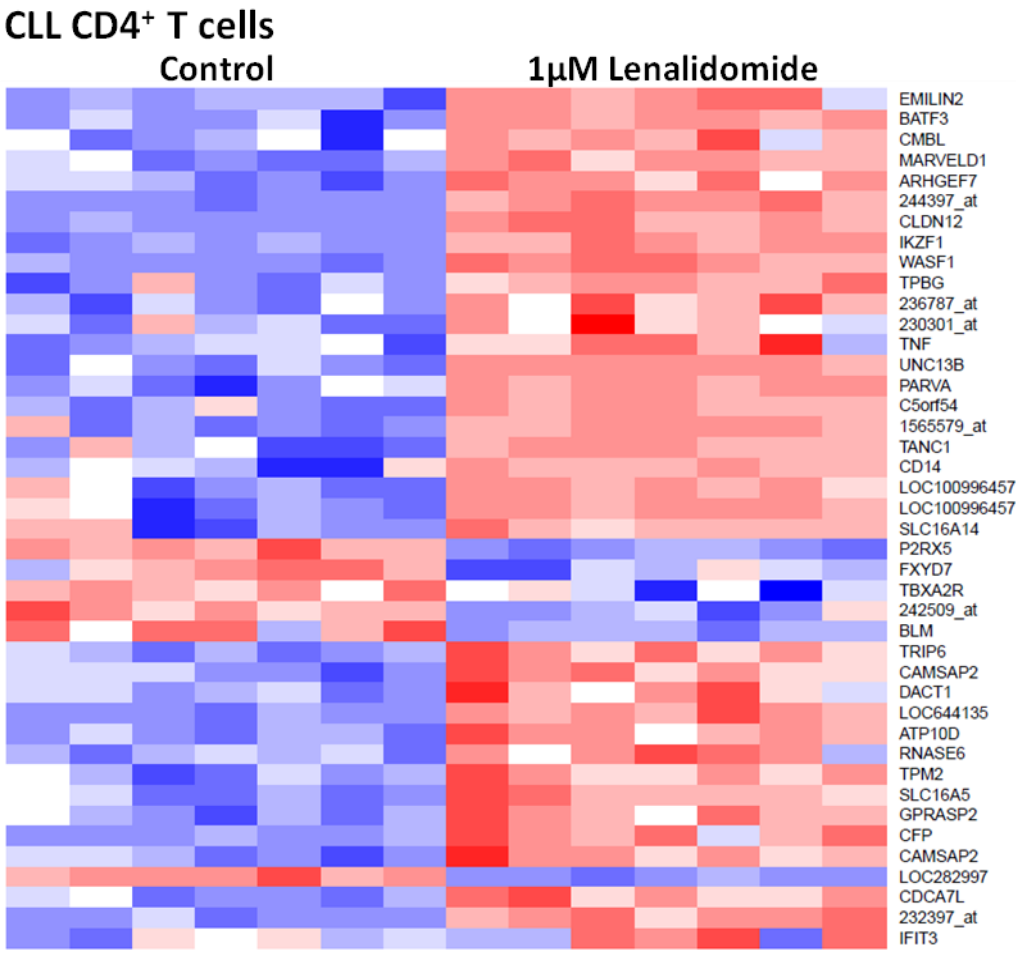
Lenalidomide treatment affected the same pathways in both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells demonstrated by alterations in the expression of common genes (**Figures 4.10**):

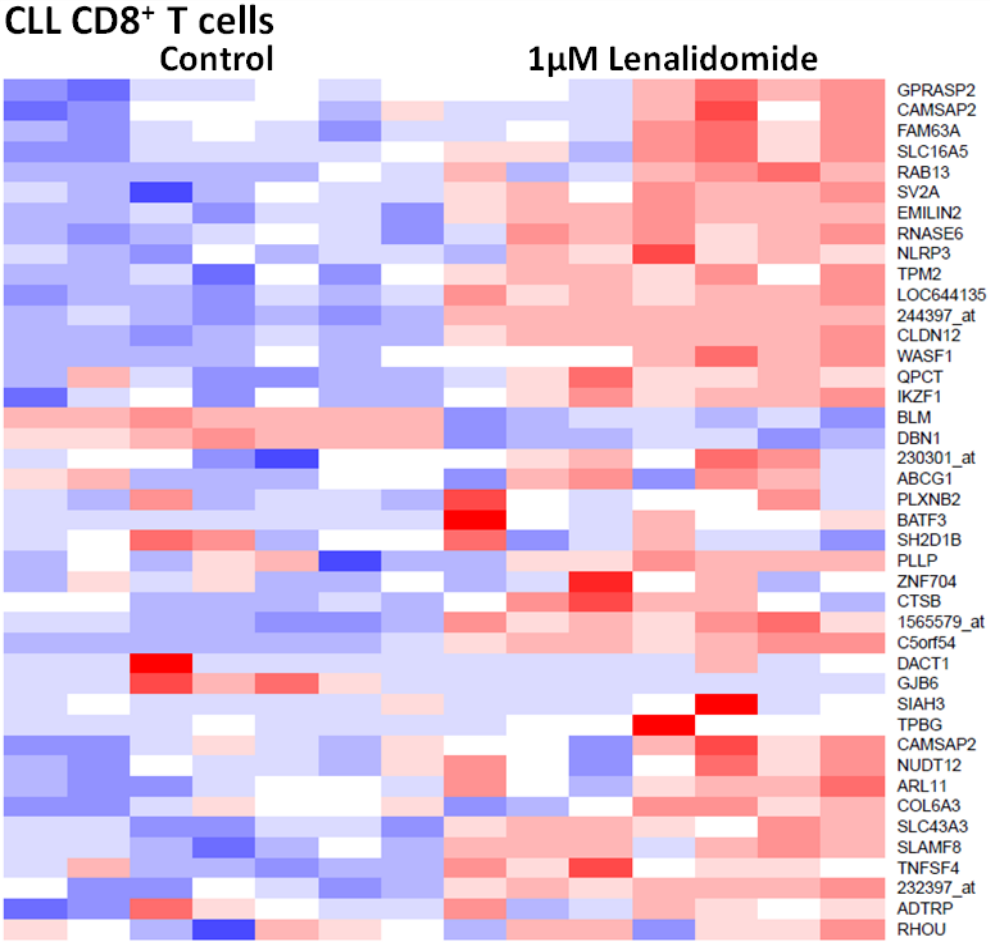
- Cytoskeletal activation: *WASF1*, *TPM2*, *RAB13*, *ARHGEF7*, *ARHGEF5*, *CAMSAP2*, *CYFIP*, *RAB20*, *RAB37*, *CORO1B*, *ARL11*
- T-cell activation: *LAG3*, *TNF*, *TNFSF4*, *IL15*
- Interferon-inducible genes: *OAS3*, *IRF4*, *IFIT3*

A list of the genes that were most affected by lenalidomide treatment ($\geq$ 3 fold change) is given in **Appendices I** and **J**.

**Figure 4.10 Heatmaps showing genes with > 3-fold change in expression after lenalidomide treatment in $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells from CLL patients**

Next page: Red = upregulated; Blue = downregulated


CLL CD4+ T cells
Control
1µM Lenalidomide
EMILIN2
BATF3
CMBL
MARVELD1
ARHGEF7
244397_at
CLDN12
IKZF1
WASF1
TPBG
236787_at
230301_at
TNF
UNC13B
PARVA
C5orf54
1565579_at
TANC1
CD14
LOC100996457
LOC100996457
SLC16A14
P2RX5
FXYD7
TBXA2R
242509_at
BLM
TRIP6
CAMSAP2
DACT1
LOC644135
ATP10D
RNASE6
TPM2
SLC16A5
GPRASP2
CFP
CAMSAP2
LOC282997
CDCA7L
232397_at
IFIT3


CLL CD8+ T cells
Control
1µM Lenalidomide
GPRASP2
CAMSAP2
FAM63A
SLC16A5
RAB13
SV2A
EMILIN2
RNASE6
NLRP3
TPM2
LOC644135
244397_at
CLDN12
WASF1
QPCT
IKZF1
BLM
DBN1
230301_at
ABCG1
PLXNB2
BATF3
SH2D1B
PLLP
ZNF704
CTSB
1565579_at
C5orf54
DACT1
GJB6
SIAH3
TPBG
CAMSAP2
NUDT12
ARL11
COL6A3
SLC43A3
SLAMF8
TNFSF4
232397_at
ADTRP
RHOU

### *4.4.12 Analysis of recurrently altered genes demonstrates that lenalidomide up-regulates "tight junction signalling" pathways in healthy and malignant lymphocytes*

The main aim of the gene expression profiling experiment was to give greater insight into lenalidomide's mechanism of action in CLL, which by extension, should allow for a greater understanding of the nature of the immune defect in this disease. Analysis of the 8 datasets showed that lenalidomide treatment altered the expression of large numbers of genes in each lymphocyte subset, in both patients and controls (**Table 4.8**).

**Table 4.8 Number of probes significantly dysregulated (>2-fold) by lenalidomide**

| No. probes | CLL $CD4^+$ T | Healthy $CD4^+$ T | CLL $CD8^+$ T | Healthy $CD8^+$ T | CLL $CD19^+$ B | Healthy $CD19^+$ B | CLL NK | Healthy NK |
|---|---|---|---|---|---|---|---|---|
| **UP** | 231 | 196 | 188 | 173 | 357 | 130 | 209 | 181 |
| **DOWN** | 47 | 38 | 65 | 35 | 173 | 107 | 62 | 54 |

However, what was immediately apparent was that the same genes kept recurring as being affected by lenalidomide treatment, whether it was in B cells, T cells, or NK cells, or from CLL patients or controls.

**Table 4.9 Top 20 genes up-regulated by lenalidomide by donor/subset**

| CLL $CD19^+$ B | Healthy $CD19^+$ B | CLL $CD4^+$ T | Healthy $CD4^+$ T | CLL $CD8^+$ T | Healthy $CD8^+$ T | CLL NK | Healthy NK |
|---|---|---|---|---|---|---|---|
| PRELP | CD1E | WASF1 | WASF1 | CLDN12 | NA | NA | NA |
| BTBD19 | RGS16 | CLDN12 | IKZF1 | NA | CLDN12 | LOC644135 | SOX4 |
| CD1C | SPARC | NA | CLDN12 | LOC644135 | WASF1 | RNASE6 | LOC644135 |
| NA | RGS16 | IKZF1 | NA | GNB4 | LOC644135 | QPCT | LOC100996539 |
| TNFRSF4 | NOD2 | LOC644135 | LOC644135 | WASF1 | NA | GNB4 | SNCA |
| IL4I1 | KCNS3 | LOC100996457 | ATP10D | IFIT3 | IKZF1 | NUDT12 | SOX4 |
| SUSD1 | CTSK | NA | RNASE6 | SLAMF8 | SLAMF8 | OAS3 | ZNF704 |
| LOC158402 | RND1 | LOC100996457 | NA | NA | RAB13 | NA | EPHA1-AS1 |
| C1orf228 | C1orf228 | ATP10D | TPBG | NA | QPCT | EMILIN2 | SOX4 |
| CCL4 | TMEM200A | RNASE6 | NA | RNASE6 | CAMSAP2 | IFIT1 | SOX4 |
| ICAM1 | NA | TPBG | NA | SLAMF8 | TNFSF4 | NLRP3 | QPCT |
| C1orf228 | TNFSF4 | NA | TNF | TPM2 | SIAH3 | SLAMF8 | NA |
| PLA2G4C | IDO1 | UNC13B | TPM2 | QPCT | CAMSAP2 | KIAA1211L | ZNF704 |
| CLDN12 | CLDN12 | NA | ARL11 | IKZF1 | PLXNB2 | C5orf54 | MFSD4 |
| SPRED2 | GPR133 | DACT1 | DACT1 | GBP4 | DACT1 | IFI44L | ERBB3 |
| MYCN | GNG8 | TANC1 | CRISPLD2 | SLC43A3 | C5orf54 | NA | GNB4 |
| RND1 | C1orf228 | ARHGEF7 | QPCT | OAS3 | ZNF704 | SNCA | KIAA1211L |
| NA | CD1E | TNF | LAG3 | GRN | COL6A3 | CYFIP1 | EPHA1 |
| IFIT3 | TJP2 | C5orf54 | C5orf54 | CYFIP1 | SLC43A3 | F2RL2 | ICA1 |
| C1orf95 | TNFRSF11B | CD14 | BATF3 | BATF3 | PLLP | C15orf48 | LTF |

Top 20 genes up-regulated by lenalidomide by donor/subset. Genes that are recurrently up-regulated in 2 or more datasets are highlighted in red.

Given that this data was generated over 2½ years using several different patients and controls, and multiple batches of lenalidomide, it was felt to be likely that these recurrently dysregulated genes were of importance. As highlighted in the table, there was the greatest degree of overlap between $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, from both CLL patients and healthy controls. Therefore it was hypothesised that lenalidomide affected a common pathway in these cells, and that focusing on genes that had altered expression in 2 or more of the T–cell datasets would aid identification of this pathway. 204 probes were significantly up-regulated (> 2-fold; adjusted *P*-value < 0.05) in 2 or more of the T-cell datasets, and 39 probes were significantly down-regulated in 2 or more of the T-cell datasets. When pathways analysis was performed on this dataset, the most differentially affected pathway was the "tight junction signalling" pathway (**Figure 4.11**).

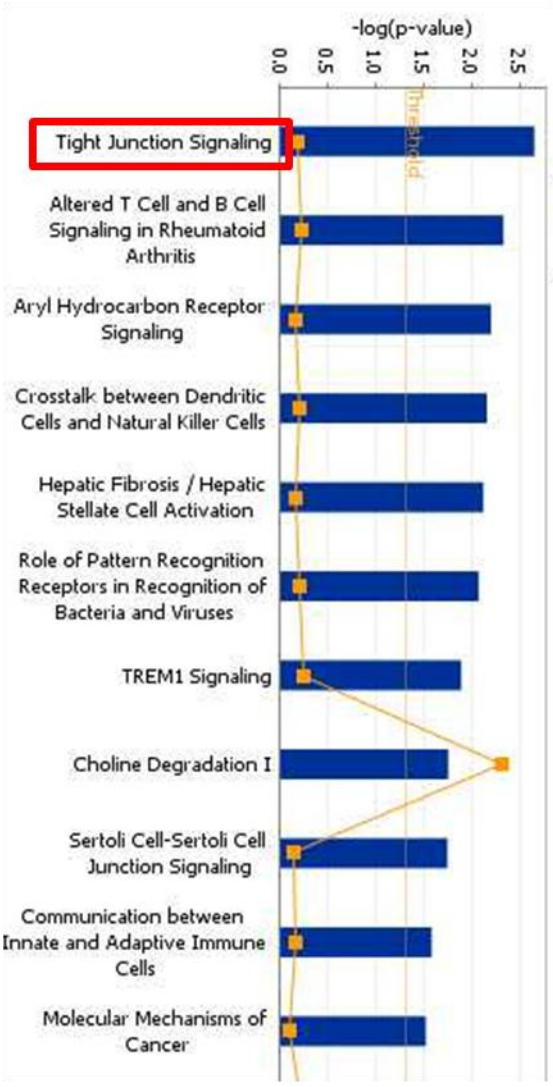



**Figure 4.11: Ingenuity analysis of recurrently altered genes identifies tight junction signalling pathway as most differentially affected by lenalidomide treatment**

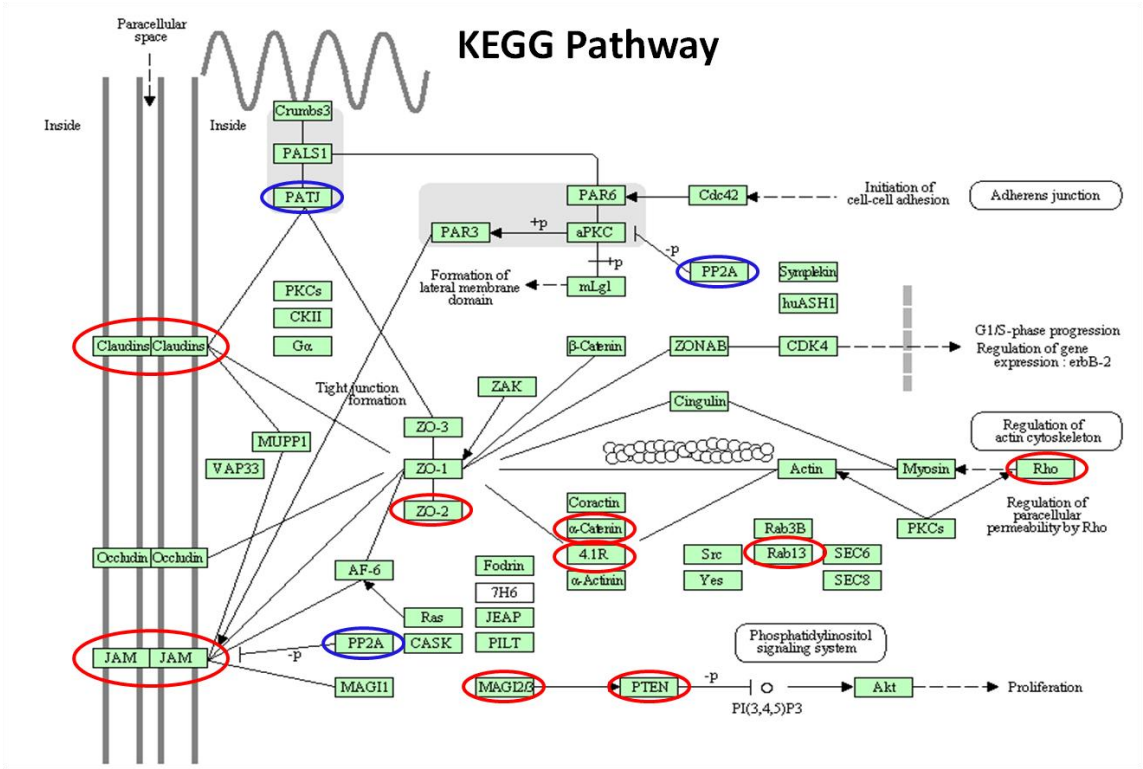



**Figure 4.12 KEGG version of tight junction signalling pathway**

Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway of tight junction signalling. The genes recurrently altered by lenalidomide are highlighted (red = up-regulated; blue = down-regulated)

**Figure 4.12** highlights the genes that were altered by lenalidomide treatment in the KEGG pathway. Genes were altered all along this pathway, from the cell membrane (e.g. *CLDN12*; Claudin12), through adaptor proteins (e.g. *TJP2*, ZO2, Tight junction protein 2) and RhoGTPases (e.g. *RAB13*, Ras-related protein Rab-13), to proteins that directly regulate the cytoskeleton (e.g. *WASF1*, Wiskott-Aldrich syndrome protein family member 1). RAB13 is known to be particularly important in tight junction signalling, and was also highlighted in the clinical study (Kohler *et al.* 2004; Chen *et al.* 2010)(see **Figure 4.4)**. Intriguingly, a gene encoding the regulatory B subunit, γ (*PPP2R3C*) of the phosphatase PP2A, which is known to be a negative regulator of tight junction signalling, was *downregulated* in this analysis. **Figure 4.13** shows the lenalidomide-induced changes in gene expression by donor subset. Importantly, genes for core tight junction (TJ) proteins, claudin12 (*CLDN12*), junctional adhesion molecule 1 (JAM1, JAM-A: *F11R*), α-catenin (*CTNNA1*), and protein 4.1 (*EPB41*), were altered across all the donor subsets. This also highlights the fact that this pathway appears to be more differentially up-regulated in T cells. A list of the genes that were recurrently affected by lenalidomide in T cells is given in **Appendices K** and **L**.

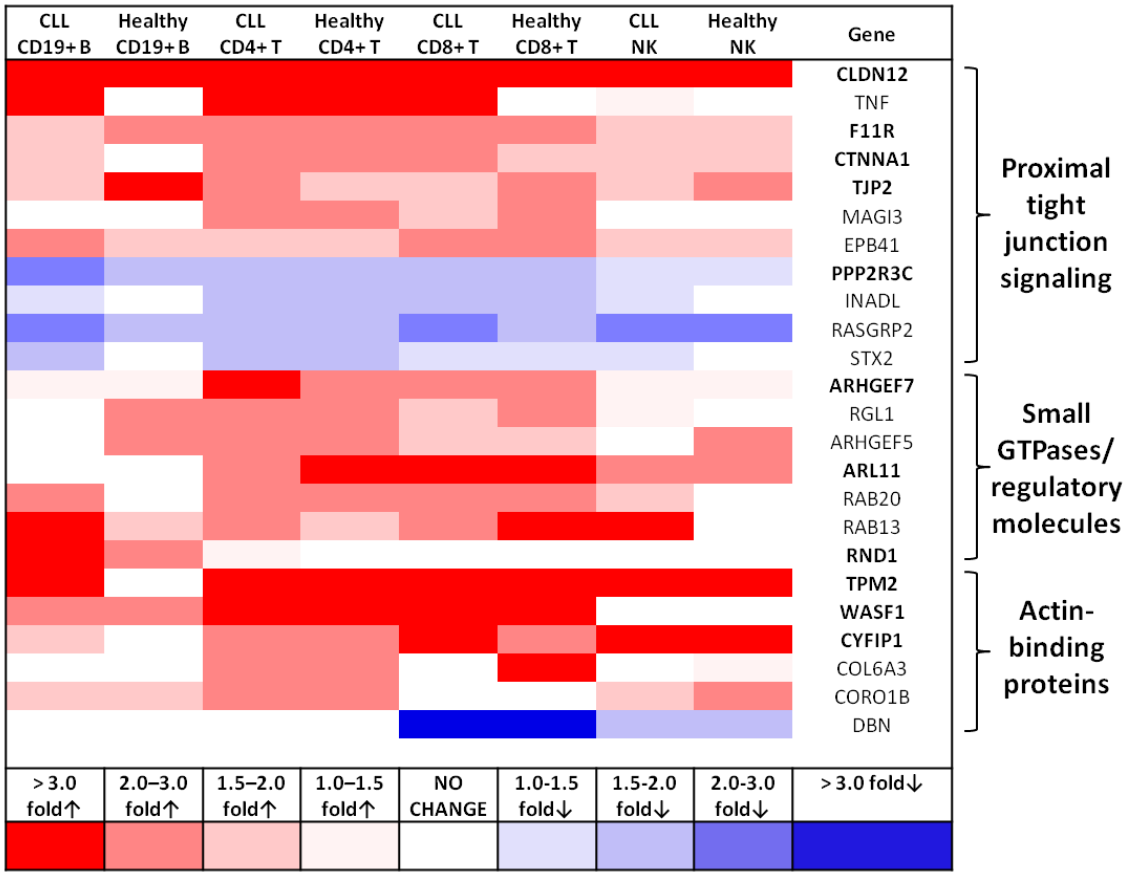



**Figure 4.13 Heatmap showing altered expression of TJ signalling genes by donor subset**

### *4.4.13 QRT-PCR validation of changes in mRNA levels of TJS genes*

The gene expression profiling data was validated at the mRNA level using QRT-PCR. As the tight junction signalling pathway was of particular interest, the genes chosen for validation purposes were related to this pathway (see **Table 4.1**; Material and Methods). The lenalidomide-induced changes in gene expression observed in the array data were all seen by PCR (**Figure 4.14**).

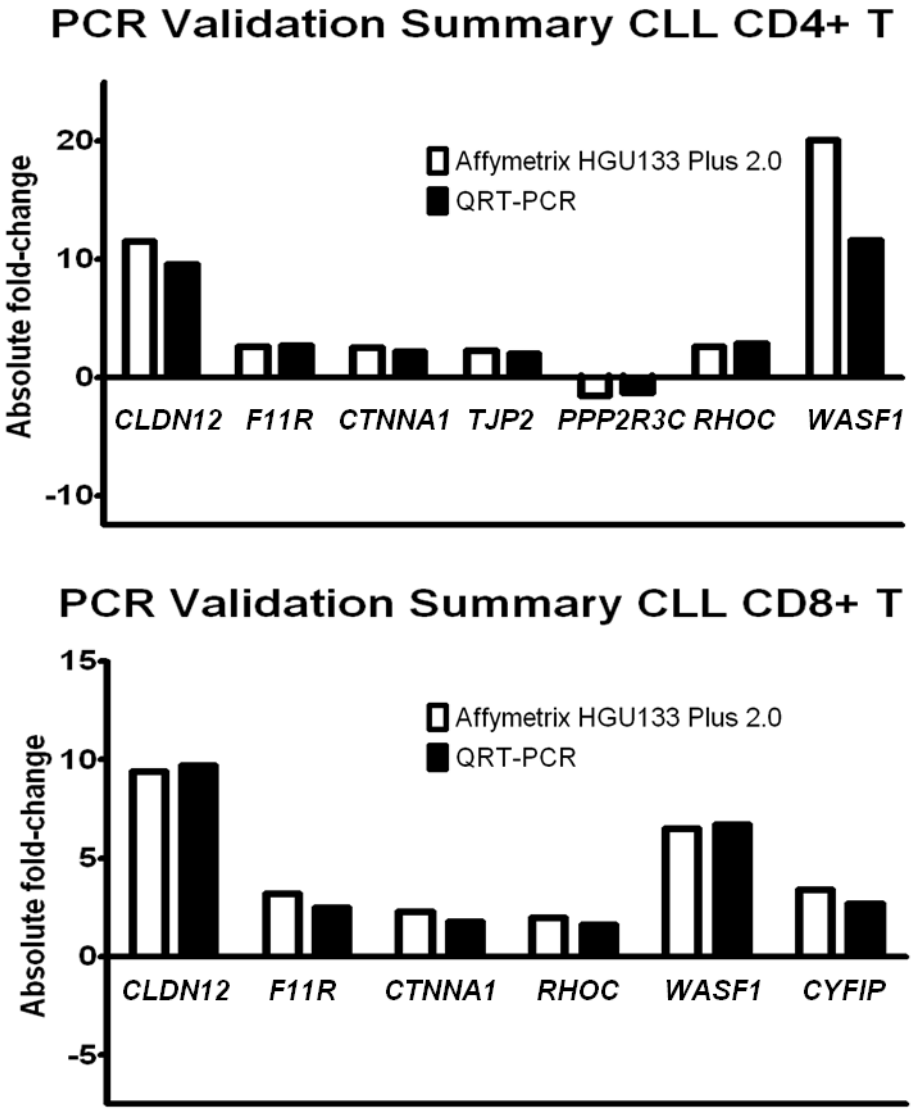



**Figure 4.14 Validation of TJ signalling gene expression changes by QRT-PCR**

### *4.4.14 Flow cytometric validation*

Further validation was performed at the protein level using flow cytometry to investigate for lenalidomide-induced upregulation of junctional adhesion molecule-A (*F11R*), which is expressed on the surface of lymphocytes. *In vitro* treatment with 1μM lenalidomide resulted in a significant increase in the MFI of F11R on CD3+CD8+ T cells from both CLL patients and healthy donors (**Figure 4.15**).

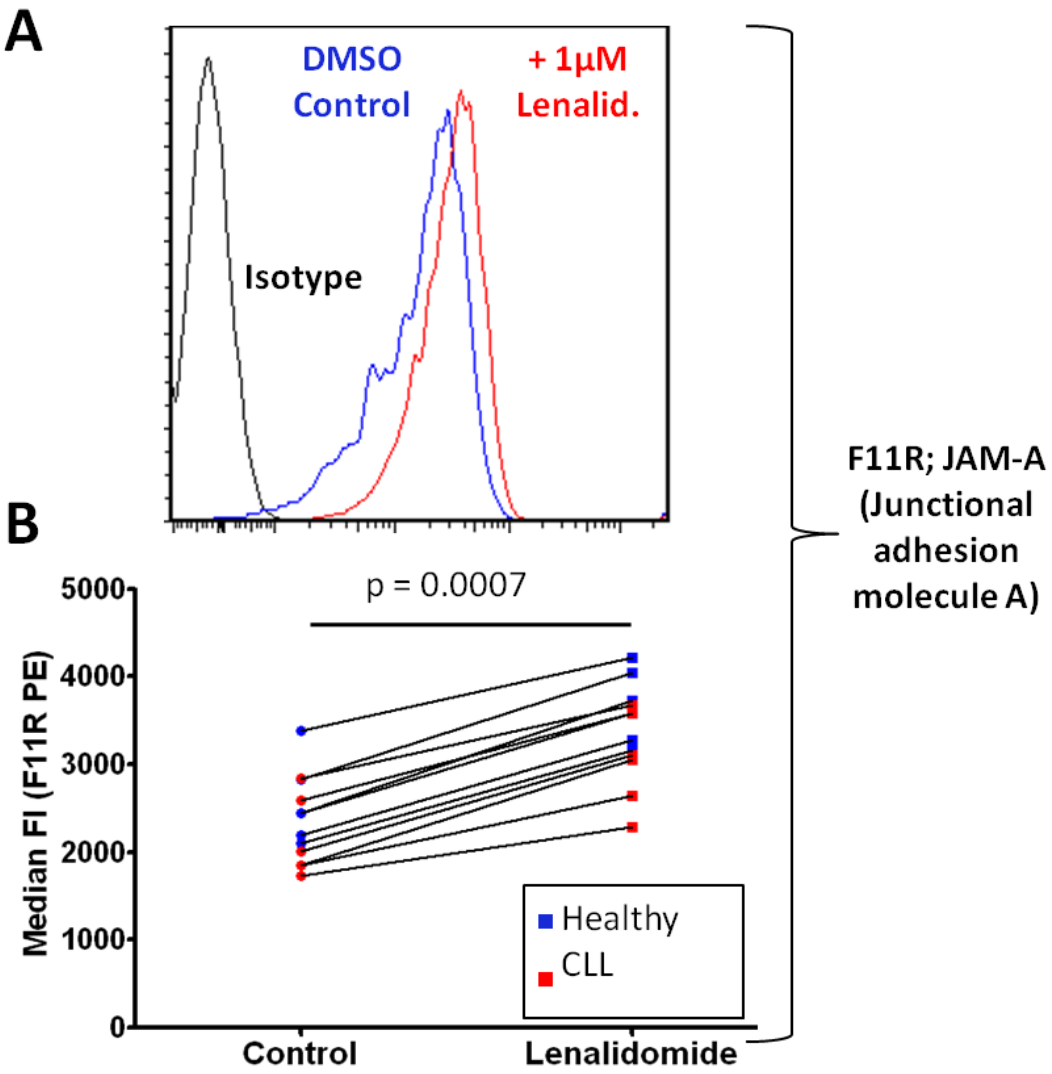



**Figure 4.15 Upregulation of F11R (JAM-A/JAM-2) on CD3+CD8+ T cells by lenalidomide**

PBMCs were treated with 1μM lenalidomide or DMSO vehicle control for 48 hours and the expression of F11R (JAM-A/JAM-2) was assessed by flow cytometry. (A) Histogram demonstrating that F11R is highly expressed by lymphocytes, and is further increased by lenalidomide treatment. (B) Lenalidomide-induced upregulation of F11R was observed on CD8+ T cells from both CLL patients (n = 6) and healthy donors (n = 6).

### *4.4.15 The impact of lenalidomide on the gene expression profiles of NK cells from CLL patients and healthy controls*

The impact of lenalidomide on the gene expression profiles of NK cells from CLL patients and healthy controls was also investigated. As before, PBMCs from CLL patients or healthy controls were cultured in the presence of 1μM lenalidomide or vehicle control for 48 hours, followed by RNA extraction and gene expression profiling. Once again, lenalidomide was an activator of gene expression with over 3 times as many probes being significantly up-regulated as down-regulated (**Table 4.8**). Some of the genes implicated in TJ signalling were also altered in NK cells, with *CLDN12*, *RAB13*, and *CYFIP* up-regulated in CLL NK cells with lenalidomide treatment, and *CLDN12*, *TJP2*, *TPM2*, *ARHGEF5*, *CORO1B*, *ARL11*, and *CYFIP* up-regulated in healthy NK cells.

However, there were also some striking differences in the effect of lenalidomide on NK cells from CLL patients when compared with healthy NK cells. In CLL NK cells, lenalidomide repaired the down-regulation of interferon-inducible genes, by increasing the expression of several of these genes including *OAS3*, *IFIT1*, *IFI44L*, *IFIT3*, *OAS1*, *F2RL*, *PDK4*, and *ACTN1*. Pathways analysis using ingenuity highlighted the effect of lenalidomide on inducing interferon signalling, and also showed significant activation of interferons α, γ, and λ as upstream regulators (**Figure 4.16**). It also highlighted the importance of upregulation of related pathways that are important in pattern recognition of bacteria and viruses, which are a crucial component of the function of NK cells in the healthy immune system.

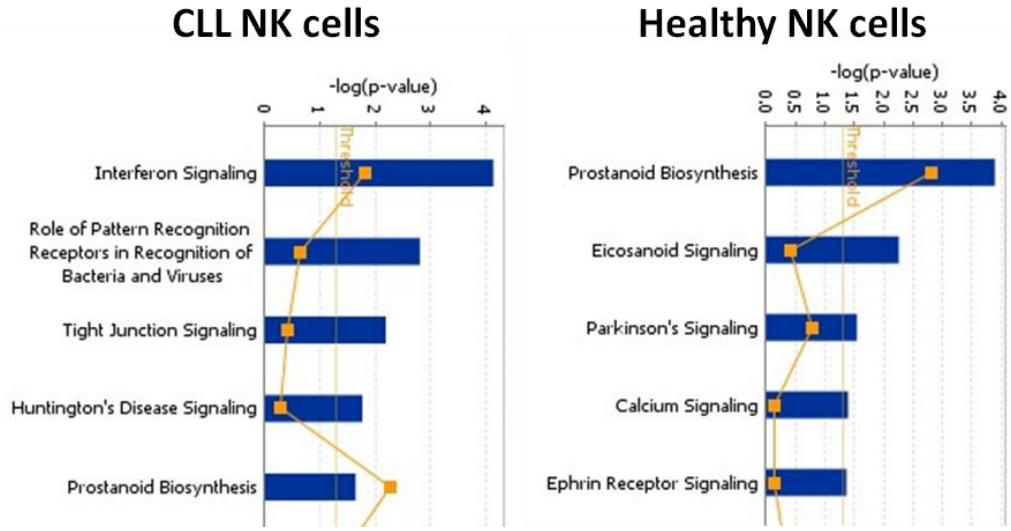



**Figure 4.16 Top 5 canonical pathways altered by lenalidomide treatment in NK cells using Ingenuity pathways analysis software**

While many of the interferon-inducible genes were up-regulated >3-fold in CLL NK cells, only *OAS3* was significantly up-regulated in healthy NK cells. Furthermore, the gene for IFNγ, *IFNG*, was actually significantly *down-regulated* in healthy NK cells by lenalidomide treatment. Furthermore, this agent also significantly down-regulated the expression of 5 genes encoding killer-cell immunoglobulin-like receptors (KIRs): *KIR2DL1*, *KIR2DL2*, *KIR2DS3*, *KIR2DS4*, and *KIR3DL2*, consistent with previous reports (Dauguet *et al.* 2010). No significant downregulation of KIR genes was observed in the CLL NK-cell dataset. Therefore, the gene expression profiling data would suggest that lenalidomide has a differential effect on CLL and healthy NK cells: in CLL NK cells it repairs defective interferon responsiveness, whereas in healthy NK cells it down-regulates inhibitory pathways. A list of the genes that were most affected by lenalidomide treatment ($\geq$ 3 fold change) is given in **Appendices M** and **N**.

## 4.5 Discussion

These results demonstrate that lenalidomide has significant effects on the gene expression profiles of lymphocytes from CLL patients and healthy controls. It has a wide variety of effects, including upregulation of cytoskeletal and interferon-inducible genes, and genes involved in B- and T-cell activation. This latter phenomenon is of particular interest in light of the fact that no cell stimulants such as anti-CD3 or BCR-cross-linking, calcium ionophores, or plant lectins, were used in the PBMC culture system, providing further evidence for a co-stimulatory effect of lenalidomide. Combined analysis of all the datasets led to the novel finding that lenalidomide activates the "tight junction signalling" pathway, with upregulation in the expression of genes throughout this pathway. This was most marked in T cells, but comparable changes were also observed in B and NK cells. Intriguingly, while there was significant overlap in the pathways affected by lenalidomide in the various lymphocyte subsets, there was also a differential effect, particularly with respect to B and NK cells, depending on whether they were from patients or controls. This may well reflect differences in the baseline state of these subsets. For example, increased of tonic BCR-signalling in CLL cells could account for some of the observed differences seen when compared with healthy B cells.

Initial concerns about the validity of using an *in vitro* culture approach were not born out. Differences in the gene expression profiles of B and T cells from CLL patients were still present after 48 hours of culture, and CLL cells treated with lenalidomide *in vitro* showed remarkably comparable changes in gene expression to those treated *in vivo* as part of a clinical trial. Furthermore, while it would have been relatively straightforward to isolate enough CLL cells for microarray analysis from CLL patients having lenalidomide treatment, it would have been extremely difficult to extract enough T or NK cells from these patients for gene expression profiling using the same method. Another advantage of this approach was that it allowed comparison of the effects of lenalidomide on lymphocytes from CLL patients and healthy individuals, as treating healthy subjects with this agent would not have been ethical.

However, there were some notable differences. In particular $CD4^+$ T cells from CLL patients showed significantly fewer dysregulated genes after 48 hours of culture than in my group's previous analysis of freshly isolated cells, whereas CLL $CD8^+$ T cells

retained their altered transcriptional state. This could be due to the fact that changes in the gene expression profiles of CD4+ T cells are induced in the lymph nodes of patients, and get normalised after 48 hours culture in PBMCs. In contrast, the changes in $CD8^+$ T cells may reflect both exhaustion, which is less readily reversible, and the fact that the PBMC culture system better reflects their "niche" in the peripheral blood of CLL patients.

Lenalidomide had profound effects on the gene expression profiles of CLL cells, with alterations in the expression of genes implicated in several pathways. Of particular interest was the finding that lenalidomide induces CLL-activation, with upregulation of genes such as *CCL4*, *LAG3*, *CD83*, *TNF*, *TNFRSF4* (OX40; CD134) and *TNFRSF13B* (TACI). This is potentially paradoxical given the recent reports of significant clinical efficacy of inhibitors of BCR-signalling such as ibrutinib and idelalisib. This observation might be explained by the fact that lenalidomide also has an activating effect on T-cells, and repairs immunological synapse formation and cytotoxicity. Activation of CLL cells may also lead to repair of their antigen presenting function rendering them more susceptible to the anti-tumour effects of the now activated and functional autologous T and NK cells. This process may be further enhanced by lenalidomide-induced secretion of the T-cell chemokine CCL4 by CLL cells, which would attract the T cells to their target.

The observations regarding the baseline differences in the transcriptome of CLL NK cells and the differential effects of lenalidomide are novel and of significant interest. NK cells from CLL patients have widespread down-regulation of interferon-inducible genes, many of which are implicated in host immunity to viral infections. It is therefore possible that decreased NK-cell responsiveness to interferon contributes to the increased susceptibility of CLL patients to intracellular pathogens such as viruses. This finding requires further investigation, in particular, whether these changes in gene expression translate into functionally defective NK-cell responses to interferon and other macrophage-derived cytokines. The differential effect of lenalidomide on NK cells from CLL patients compared to healthy controls is also intriguing. While lenalidomide up-regulates interferon inducible genes and could be postulated to restore NK-cell functionality in CLL, it has more complex effects on NK cells from healthy individuals, down-regulating both IFNγ and the expression of inhibitory KIR molecules.

The finding that lenalidomide activates tight junction (TJ) signalling in lymphocytes, particularly in T cells, was unexpected. Tight junctions are closely associated areas of two epithelial or endothelial cells whose membranes join together forming a barrier to fluid (**Figure 4.17)**. Their principle functions are to hold cells together, joining the cytoskeletons of adjacent cells maintaining apical-basal cell polarity, and to prevent the passage of molecules and ions through the intercellular space. This latter function is particularly important for epithelial layers, for example in the gut, where trans-cellular route exchange allows for control of ion and molecular gradients (Matter and Balda 2003).

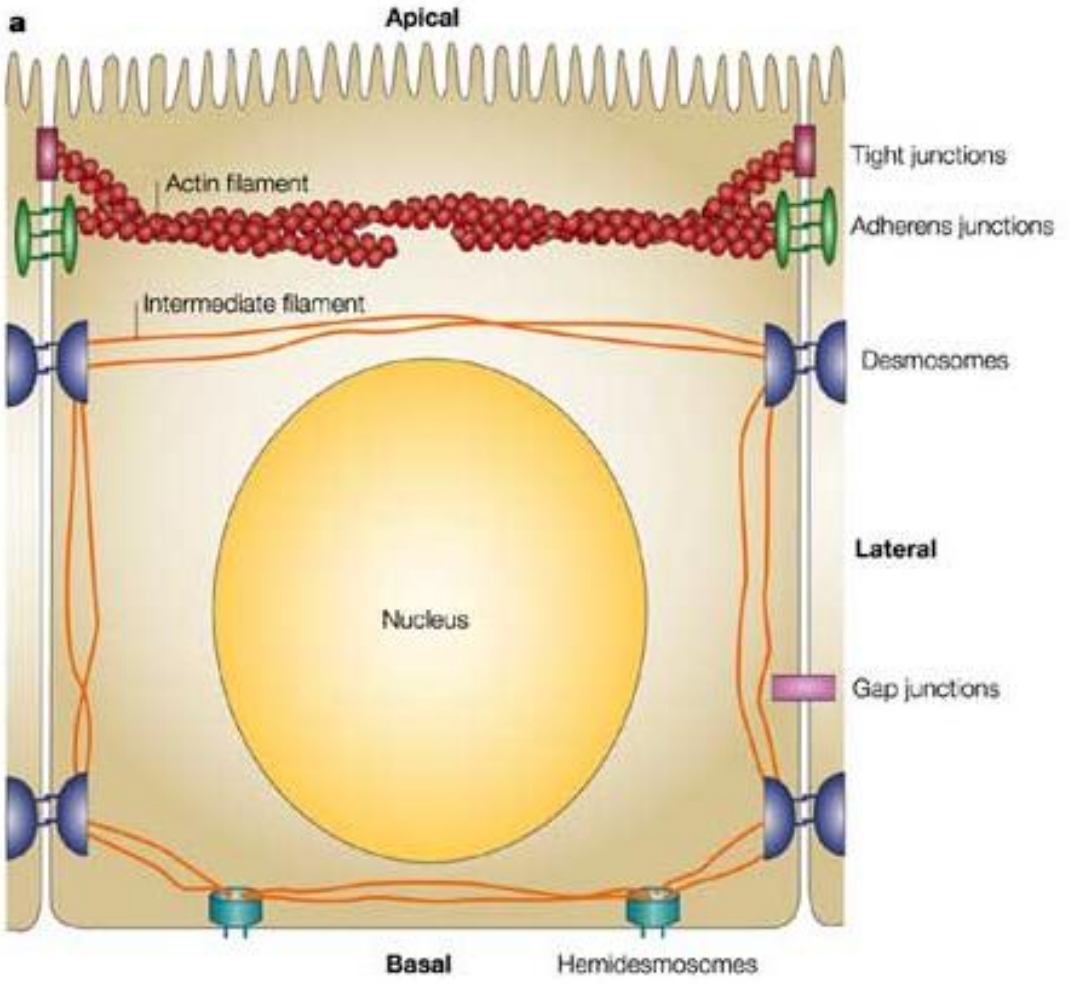



**Figure 4.17 Epithelial intercellular junctions**

A schematic representation of a polarised absorptive epithelial cell. The different types of intercellular junction, as well as hemi-desmosomes, a type of cell-extracellular matrix adhesion a shown. It should be noted that TJs and adherens junctions (AJs) are linked to the actin cytoskeleton, whereas desmosomes and hemidesmosomes are linked to intermediate filaments (Matter and Balda 2003).

TJs were long regarded as simple barriers that separated compartments of different composition, but more recent research has also implicated them in the regulation of cell signalling. Evidence suggests that these structures are involved in receiving and converting signals from the cell interior that regulate their assembly and function ("inside-out"), as well as transmitting signals to the cell interior to modulate gene expression and cell proliferation ("outside-in"). This is an emerging area, but several pathways of significant interest in haematological malignancies have been implicated, including cyclin D1, myc, HDAC and AP1 (**Figure 4.18**).

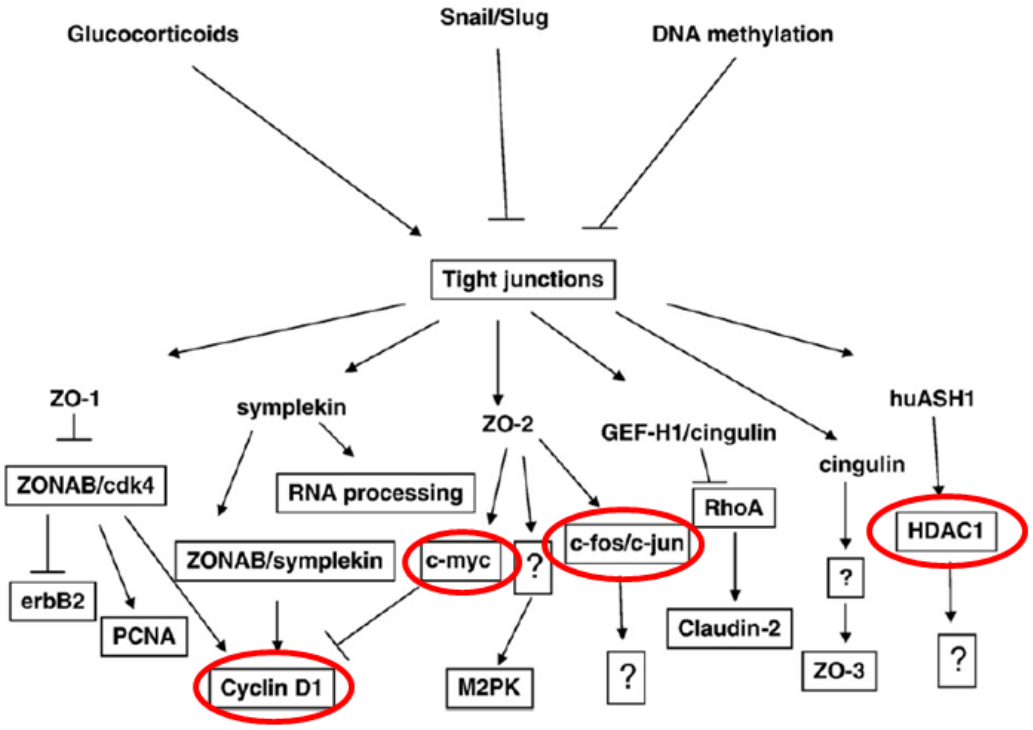



**Figure 4.18 TJ proteins in the control of gene expression (Balda and Matter 2009)**

In light of lenalidomide's known effects on actin polymerisation and RhoGTPase activation, the close association of TJ signalling to the cytoskeleton is of obvious interest (Ramsay *et al.* 2008; Xu *et al.* 2009). RhoGTPases such as Rac, RhoA and cdc42 are important components of TJs, and are known to be activated by lenalidomide. The link between ZO2 (TJP2; tight junction protein 2) and the AP1 transcription factors c-fos and c-jun is also of interest, given reports that co-stimulatory activity of IMiDs is associated with increased AP1 activity (Schafer *et al.* 2003). The next chapter will investigate TJ signalling in lymphocytes, and also examine whether an understanding of the regulation of TJ signalling illuminates the mechanism of action of lenalidomide.

# Chapter 5: The mechanism of action of lenalidomide

## 5.1 Introduction

### *5.1.1 Tight junction signalling in lymphocytes*

Investigation of the impact of lenalidomide on the gene expression profiles of healthy and malignant lymphocytes demonstrated that it activates the tight junction (TJ) signalling pathway, particularly in T cells. Very little is known about the role of this pathway in lymphocytes, although downstream molecules such as the RhoGTPases Rac1, RhoA, and cdc42 are known to be crucial, both in assembly/disassembly of TJs and in lymphocyte function. The junctional adhesion molecules JAMA, JAMB, and JAMC are known to be highly expressed by various lymphocyte subsets, and there are reports linking expression of these molecules to lymphocyte function and differentiation. JAMC has been demonstrated to be up-regulated in activated T cells via a transcriptional mechanism, suggesting a potential role for this molecule in T cell function (Immenschuh *et al.* 2009). JAMC has also been implicated in B-cell differentiation as its expression on $CD27^+$ B cells has been shown to delineate B cells that have not undergone a germinal centre reaction (Ody *et al.* 2007). Occludin has also been shown to be up-regulated on activated lymphocytes, whereas it is virtually absent on resting T cells (Alexander *et al.* 1998). Interestingly, expression of claudin 1 and claudin 5 are elevated on peripheral blood leucocytes in multiple sclerosis patients at relapse, with claudin 5 and JAMC mRNA levels falling after glucocorticoid treatment (Mandel *et al.* 2012). There is plenty of evidence implicating these molecules in the transendothelial migration of leucocytes, but nearly all of it has focused on the expression of these molecules by the endothelial cells rather than the leucocytes (Ostermann *et al.* 2002). However, other investigators have highlighted the high expression of junctional adhesion molecules by lymphocytes, and have suggested that there expression *on leucocytes* may play a role in transendothelial migration, potentially via homophilic interactions (Muller 2003). Interestingly, components of the tight junction pathway are known to be important for platelet aggregation; indeed JAMA was first described as an activating platelet receptor (F11R)(Gupta *et al.* 2000).Therefore, it is possible that activation of this pathway underlies the increased risk of thrombosis seen with the clinical use of IMiDs.

It light of this, it was hypothesised that components of this pathway may play an important role in lymphocyte function, with aspects of TJ signalling being involved in cell motility and immunological synapse formation, accounting for the functional effects that my group has previously described. Therefore, the aim of the first part of this chapter was to assess whether there is any evidence of defective TJ signalling in lymphocytes from CLL patients.

### *5.1.2 The regulation of tight junction signalling*

The second aim of this chapter was to examine whether an understanding of the regulation of TJ signalling illuminates the mechanism of action of lenalidomide. TJs are a very active part of epithelial/endothelial cell layers, with high plasticity. Assembly and disassembly of TJs is a continuous process, and can occur by endocytotic internalization of TJ proteins or by supply of new TJ proteins from the Golgi apparatus. Occludin was the first integral TJ protein identified, and a consequence its regulation has been the most extensively studied. It localisation within the cell depends on its phosphorylation status, which is tightly regulated. Several kinases have been implicated in this process, including aPKC, PKA, MAPK, PI3K, CK1, CK2 and RhoK (Schulzke *et al.* 2012). Phosphatases are understandably also implicated, with PP2A of particular importance as a negative regulator. PP2A over-expression severely prevents TJ assembly, whereas inhibition of PP2A by okadaic acid promotes the phosphorylation and recruitment of ZO1 (zona occludens 1; tight junction protein 1), occludin and claudin1 to the TJ during functional biogenesis. The inhibitory effect of PP2A is mediated by its ability to dephosphorylate aPKCζ at the regulatory Thr410 residue, blocking the activation of this kinase (Nunbhakdi-Craig *et al.* 2002). PP2A also inhibits TJ signalling by directly dephosphorylating JAMA at Ser285, blocking JAMA adhesion and the development of a functional epithelial barrier (Iden *et al.* 2012).

PP2A is one of the four major serine/threonine phosphatases. Like most phosphatases its broad substrate specificity means that it is implicated in many cellular processes, in particular as a regulator of cell growth and division. It is a heteromeric core enzyme composed of a structural A subunit, a regulatory B subunit, and a catalytic C subunit.

Notably, there is evidence linking PP2A to the activity of lenalidomide in MDS. An early clinical study found that patients with 5q deleted MDS responded especially well to lenalidomide compared to patients with a normal karyotype or other karyotypic

abnormalities (List *et al.* 2005). This observation sparked a great deal of interest in whether the genes that were normally encoded in the deleted part of chromosome 5q were important in the mechanism of action of lenalidomide. As a result, several candidate genes were identified as being potentially important in lenalidomide sensitivity, which included PP2A, which has two subunits encoded on chromosome 5q, and a gene encoding another component of the TJ signalling pathway, α-catenin (Padron *et al.* 2011). Subsequent work showed that PP2A was inhibited by lenalidomide. This provided an explanation for the clinical efficacy of this drug, as further inhibition of PP2A in 5q deleted MDS progenitors that were already haplo-deficient for this phosphatase, made them especially susceptible to cell arrest (Wei *et al.* 2009).

Other investigators have also noted a link between PP2A inhibition and lenalidomide. Lenalidomide has been shown to promote expression of CD154 on CLL cells through a PI3K-dependent mechanism (Lapalombella *et al.* 2010). Further work demonstrated that this effect is mediated by PP2A inhibition, which results in PI3K activation and increased AKT phosphorylation. This is supported by observations that the PP2A activating agent 1,9-di-deoxyforskolin, when co-incubated with lenalidomide, antagonises lenalidomide induced CD154 mRNA and protein, whereas treatment with the PP2A inhibitor okadaic acid mimics the effects of lenalidomide on CD154 and AKT phosphorylation. Therefore assessment of the effect of lenalidomide on the activity of PP2A in CLL cells and T cells was the second aim of this chapter.

## 5.2 Materials and Methods

### *5.2.1 Patients and Samples*

Peripheral blood samples were obtained from CLL patients from the tissue bank maintained by the Department of Haemato-Oncology of St. Bartholomew's Hospital, London. Ethical approval was confirmed by the East London & The City Health Authority Local Research Ethics Committee, and written informed consent was obtained in accordance with the Declaration of Helsinki. Healthy PBMCs were obtained from buffy coats/cones supplied by the National Blood Service. The same RNA samples that were used for the vehicle control condition for the gene expression profiling were also used for QRT-PCR. A different cohort of samples was used for the flow cytometric analyses.

### *5.2.2 Cell Culture and isolation of lymphocyte subsets*

Peripheral blood samples were cultured as described in section 4.3.2. The lymphocyte subsets were isolated and their purity determined as described in sections 4.3.3 and 4.3.4.

### *5.2.3 QRT-PCR*

The expression of six genes involved in TJ signalling was compared between CLL cells and healthy B cells, and between T cells from CLL patients and controls (**Table 5.1**). RNA extracted from the vehicle control samples (i.e. lenalidomide untreated) was re-used for the analysis. 8 patients and controls were used for $CD8^+$ T cells and B cells, and 7 patients and controls were used for $CD4^+$ T cells. In all cases the expression of each target was normalised to the expression of GAPDH (Human GAPDH endogenous control; Invitrogen/Applied Biosystems) to give a $\Delta C_T$ value. This was calculated by subtracting the mean $C_T$ value for reference gene (GAPDH) from the mean $C_T$ value of the target gene. As the fluorescence doubles with each cycle during the exponential phase of the reaction, log base 2 of the $-\Delta C_T$ value ($2^{-\Delta CT}$) gives a measure of the absolute relative expression of each gene compared to GAPDH in each sample. This value was then multiplied by 100 ($100 \times 2^{-\Delta CT}$) to give % expression of each target gene relative to GAPDH.

**Table 5.1 Taqman primers used for QRT-PCR**

| Gene Name | Description | Taqman® Gene Expression Array |
|---|---|---|
| *CLDN12* | claudin 12 | Hs01082669_m1 |
| *F11R* | F11 receptor | Hs00170991_m1 |
| *CTNNA1* | catenin (cadherin-associated protein), alpha 1, 102kDa | Hs00944794_m1 |
| *TJP2* | tight junction protein 2 (zona occludens 2) | Hs00910543_m1 |
| *RND1* | Rho family GTPase 1 | Hs00205507_m1 |
| *WASF1* | WAS protein family, member 1 | Hs01591751_m1 |

### *5.2.4 Monoclonal antibodies*

The following directly conjugated monoclonal antibodies were used in this study: CD40-PECy7, CD80-PECy7, CD83-PE and CD321-PE were all obtained from BD Biosciences; CD5-PerCPCy5.5, CD19-APCCy7, CD3-Pacific-Blue, CD8-PerCpCy5.5, and IFNγ-FITC were all obtained from eBioscience.

### *5.2.5 Immunofluorescence staining and flow cytometric analysis*

Surface and intracellular staining for flow cytometric analysis was performed as described in section 2.7. Flow cytometry was performed on a BD Fortessa flow cytometer with subsequent analysis using FlowJo software (Tree Star). Analysis was performed on a minimum of 10,000 events after gating on live singlet cells.

### *5.2.6 Cell stimulation and lenalidomide/okadaic acid treatment*

For assessing the impact of lenalidomide or okadaic acid on cell surface molecule expression, PBMCs were cultured for 48 hours in the presence of 1μM lenalidomide, 10nM okadaic acid or 0.01% DMSO vehicle control. For assessment of IFNγ production PBMCs or purified $CD3^+CD8^+$ T cells were stimulated with 1μg/ml soluble anti-CD3 (clone HIT3a, BD) and 5μg/ml soluble anti-CD28 (clone CD28.2, eBioscience) for 72 hours and incubated at $37^oC$; 5% $CO_2$. 4 hours before analysis 0.66 μl/ml Golgistop (BD) was added to inhibit cytokine secretion.

### *5.2.7 Western blotting*

Western blotting was performed as described in section 2.11.3. Polyclonal primary rabbit antibodies to human ZO2 (TJP2) and α-catenin were from Cell signaling (New England Biolabs); rabbit polyclonal antibody to human claudin12 was from invitrogen. The goat anti-rabbit-HRP secondary antibody was from Dako.

### *5.2.8 Statistical analysis*

All data sets were subject to normality testing using the Shapiro-Wilk normality test. Where all data sets could be accurately modelled by a Gaussian distribution an unpaired t test was used for analysis of differences between groups; where this was not the case the 2-sided Mann Witney *U* test was used. For comparison of more than two groups the Kruskal-Wallis test was used with Dunn's post test for multiple comparisons. *P* values of less than 0.05 were considered statistically significant.

## 5.3 Results

### *5.3.1 Junctional adhesion molecule-A (JAMA) is down-regulated on CD8$^+$ T cells from CLL patients*

In light of the observations regarding up-regulation of TJ signalling by lenalidomide, it was hypothesised that some of the clinical efficacy of this agent in CLL might be due to repair of this pathway which is in turn defective in patient T cells. Junctional adhesion molecule-A (JAMA; JAM1; F11R) is 32-35kDa protein that is expressed on platelets, leukocytes, red blood cells, endothelial cells, epithelial cells, and various cell lines. It is known to be important in platelet function and is expression on *endothelial cells* is has been demonstrated to be crucial for leucocyte transendothelial migration (Ostermann *et al.* 2002). Despite the fact that JAMA is known to be highly expressed by lymphocytes, relatively little is known about its function on these cells. The levels of JAMA mRNA and protein were compared between CD8$^+$ T cells from CLL patients and healthy controls. CLL CD8$^+$ T cells were observed to have decreased levels of JAMA mRNA by QRT-PCR ($P < 0.0001$). Patient CD8$^+$ T cells were also noted to have reduced expression of JAMA protein on their surface ($P < 0.0001$), which this defect becoming more pronounced with disease progression (**Figure 5.1**).

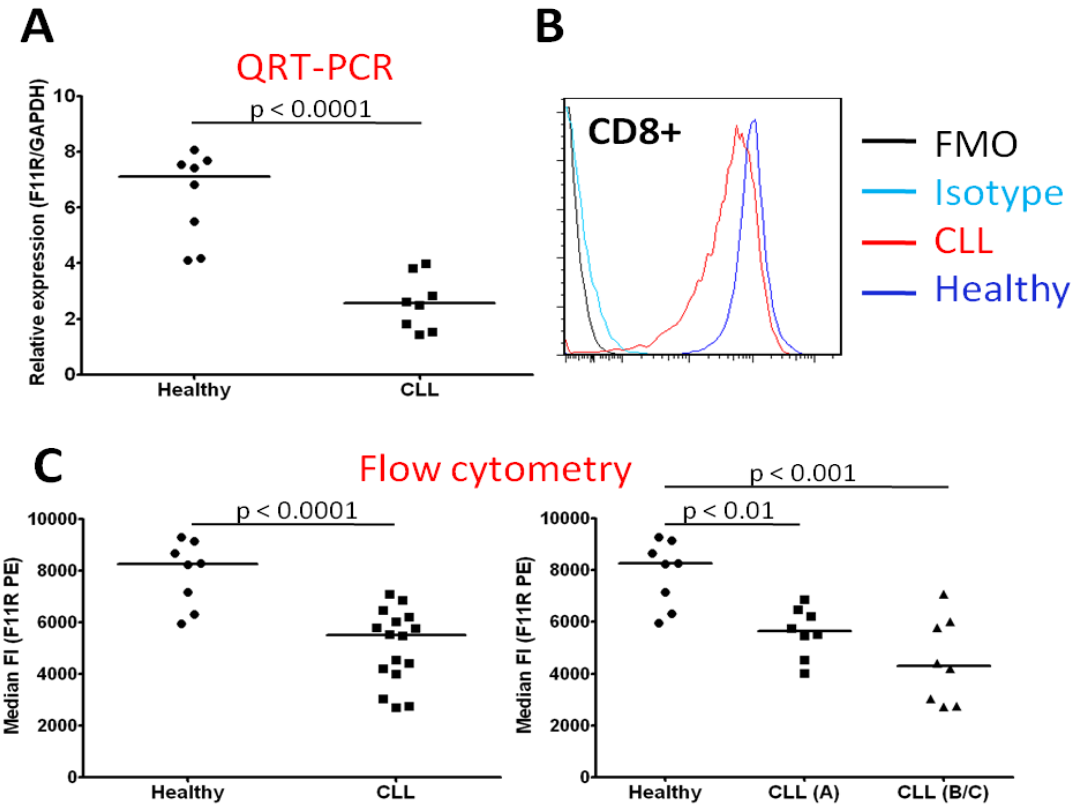



**Figure 5.1 JAMA (F11R) is downregulated on CD8$^+$ T cells from CLL patients**

(A) Levels of JAMA mRNA are significantly reduced in CLL CD8$^+$ T cells. (B)&(C) The expression of JAMA protein is reduced on CLL CD8$^+$ T cells and falls further with disease progression (Binet B/C)

### *5.3.2 Levels of JAMA mRNA are reduced in CD4$^+$ T cells from CLL patients but this does not translate into significantly reduced protein expression*

The expression of JAMA was also compared on CD4$^+$ T cells from CLL patients and healthy controls. As with CD8$^+$ T cells there was a significant reduction in the levels of JAMA mRNA by QRT-PCR ($P$ = 0.0006). However this did not translate in to significant reduction in protein expression on the cell surface as measured by flow cytometry ($P$ = 0.15)(**Figure 5.2**).

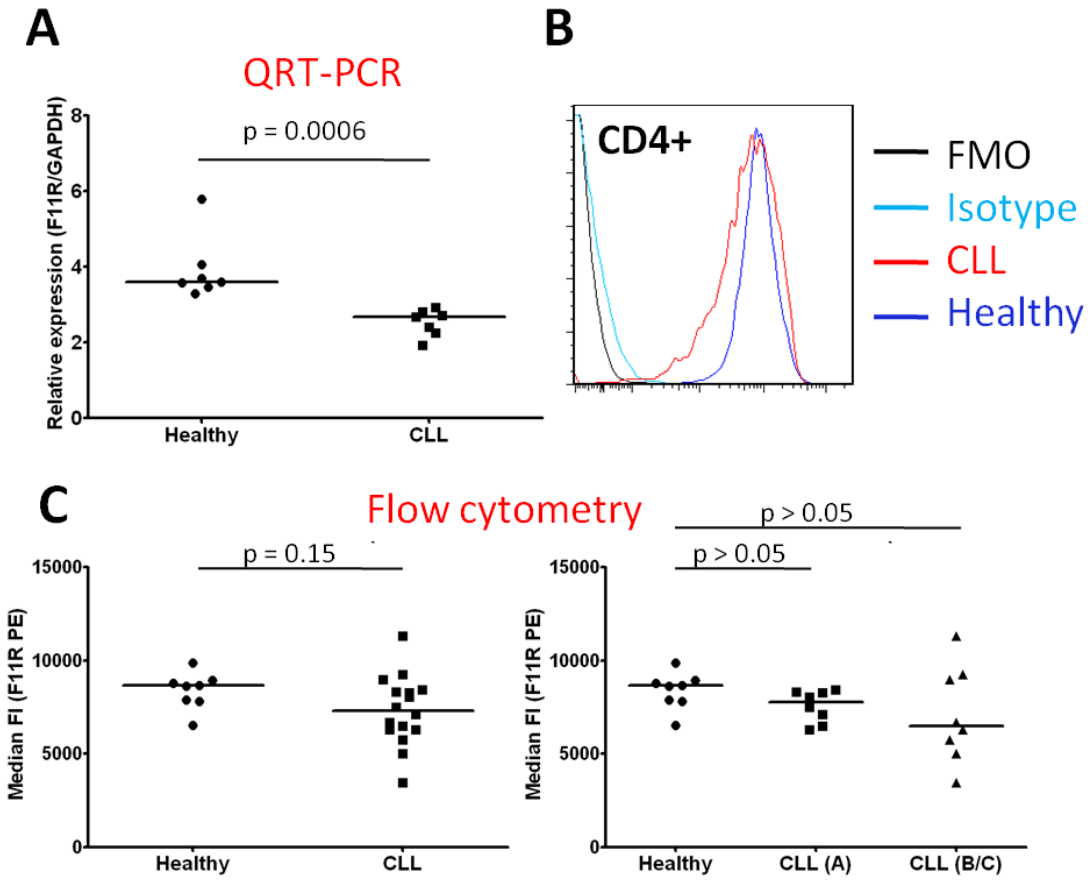



**Figure 5.2 JAMA mRNA is down-regulated in CD4$^+$ T cells from CLL patients**

(A) Levels of JAMA mRNA are significantly reduced in CLL CD4$^+$ T cells. (B)&(C) The expression of JAMA protein is not significantly reduced on CLL CD4$^+$ T cells

### *5.3.3 The expression of proximal TJ signalling genes are downregulated in CD8$^+$ T cells*

The expression of the proximal TJ signalling genes *CLDN12* and *TJP2*, and the cytoskeletal gene *WASF1* was compared between CD8$^+$ T cells from CLL patients and healthy donors. There was a significant decrease in the expression of these genes in CD8+ T cells from CLL patients (**Figure 5.3**).

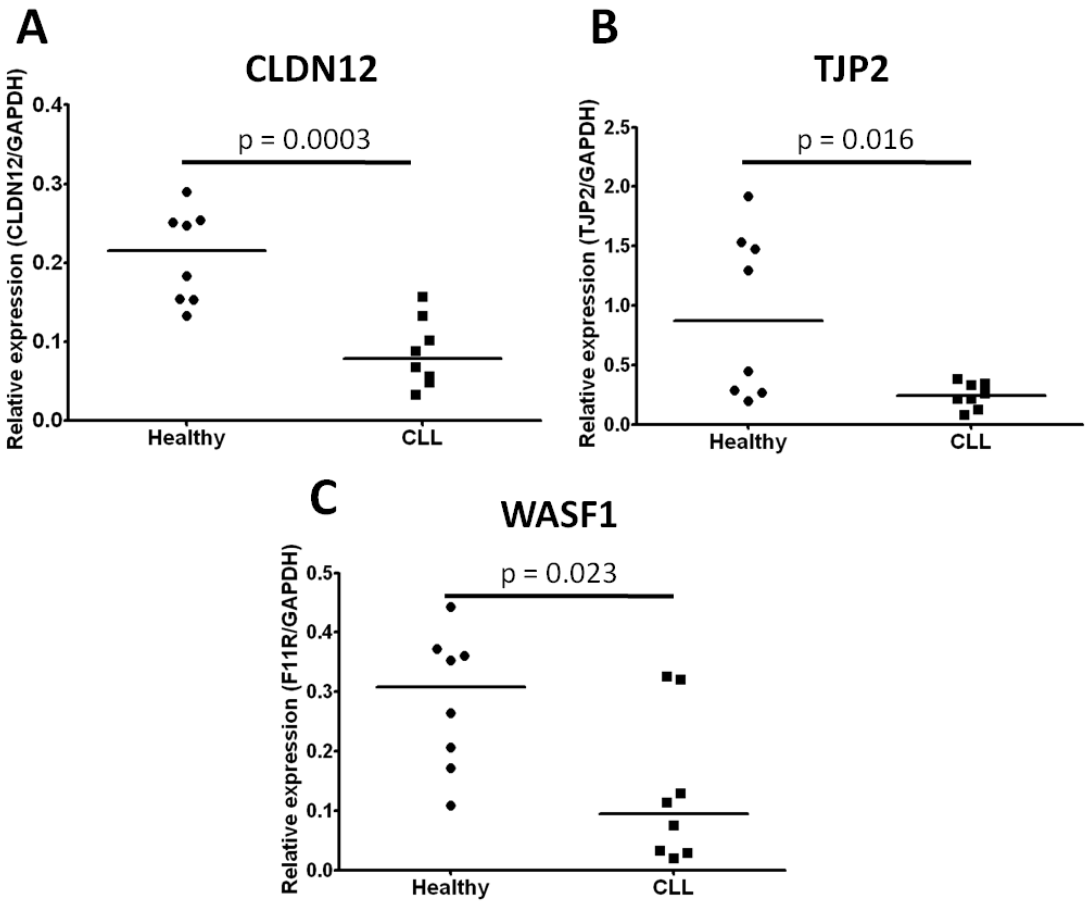



**Figure 5.3 The expression of proximal TJ genes is down-regulated in CD8$^+$ T cells from CLL patients**

The expression of *CLDN12*, *TJP2*, *WASF1* was compared by QRT-PCR. There was decreased expression of *CLDN12* (A), *TJP2* (B), and *WASF1* (C) in CD8+ T cells from CLL patients.

### *5.3.4 The expression of proximal TJ signalling genes in CD4$^+$ T cells*

The expression of the proximal TJ signalling genes *CLDN12* and *TJP2*, and the cytoskeletal gene *WASF1* was also compared between CD4$^+$ T cells from CLL patients and healthy donors. While there was a significant decrease in the expression *TJP2* (*P* = 0.039) there was no change in the expression of *CLDN12* or *WASF1* (**Figure 5.4**). Overall, while there is some evidence consistent with a defect in this pathway in CD4$^+$ T cells from CLL patients, this is not a marked as in CD8$^+$ T cells

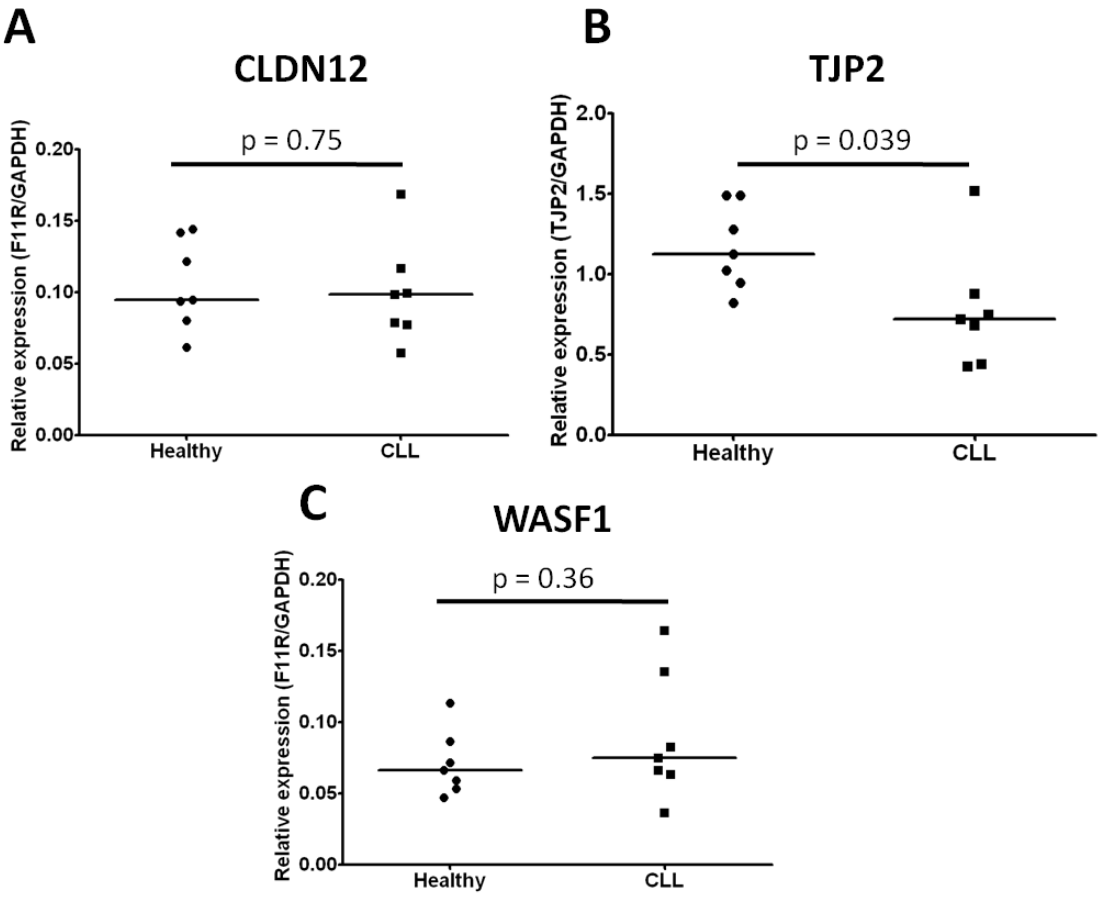



**Figure 5.4 The expression of proximal TJ signalling genes in CD4+ T cells**

The expression of *CLDN12*, *TJP2*, *WASF1* was compared by QRT-PCR. There was decreased expression of *CLDN12* (A), *TJP2* (B), and *WASF1* (C) in CD8+ T cells from CLL patients.

### 5.3.5 JAMA is up-regulated on CLL cells

Previous work by my group demonstrated that treatment of both CLL cells and autologous T cells with lenalidomide was required to repair dysfunctional immunological synapse formation (Ramsay *et al.* 2008). This suggested that a cytoskeletal defect was also present in CLL cells, which can be repaired by lenalidomide. In light of this the expression of components of TJ signalling were also compared between CLL cells and healthy B cells. Surprisingly, there were increased levels of JAMA mRNA in CLL cells, which correlated with increased expression of JAMA protein on the cell surface (**Figure 5.5**). These findings also validated data from the Affymetrix array comparison between healthy and CLL B cells, which also found increased expression of the genes that encodes JAMA, *F11R*.

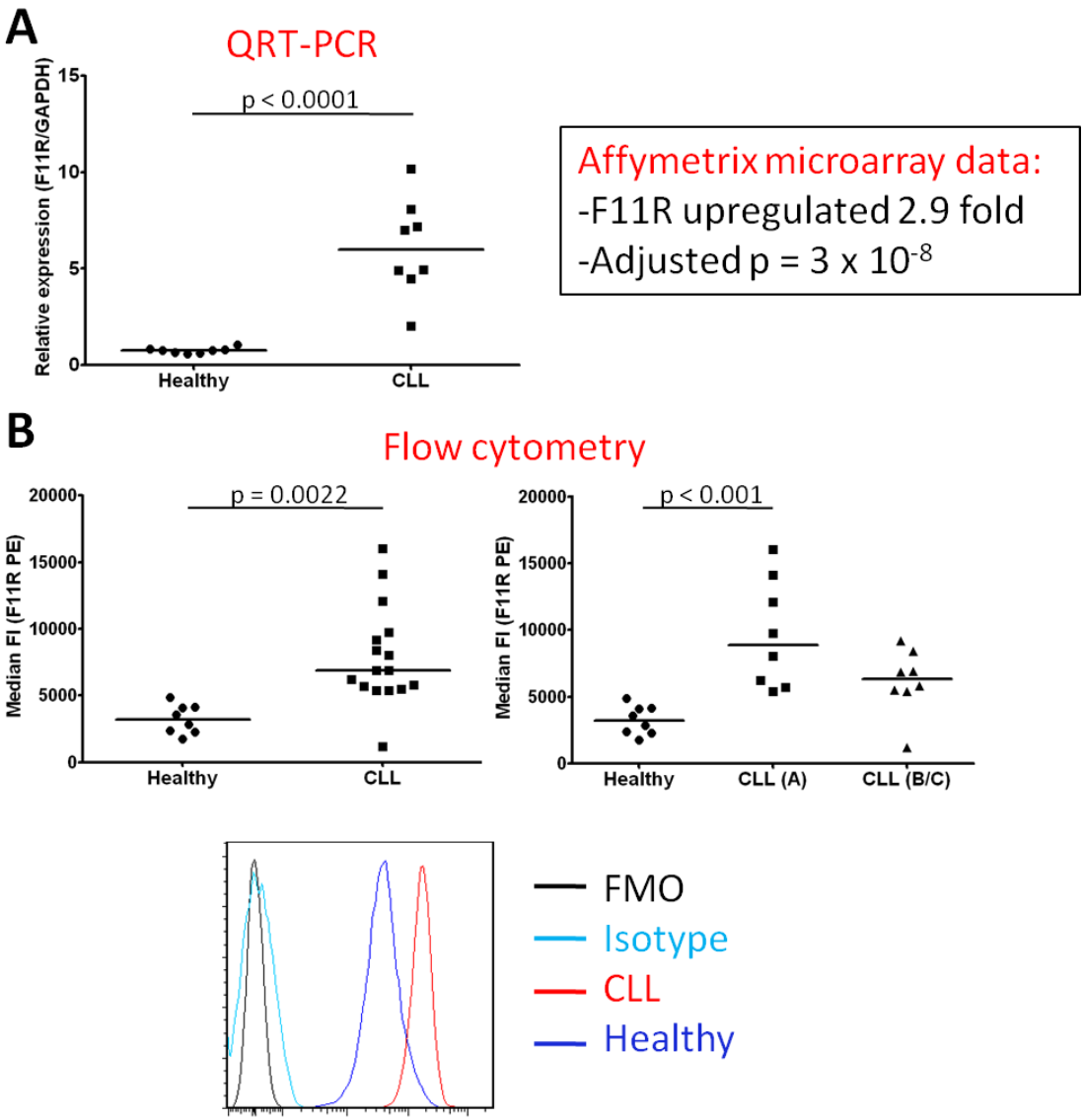



**Figure 5.5 JAMA is up-regulated on CLL cells**

(A) Increased expression of JAMA mRNA in CLL cells by QRT-PCR and from the Affymetrix microarray dataset. (B) Increased expression of JAMA by flow cytometry

### *5.3.6 Increased expression of proximal TJ signalling genes in CLL cells*

The expression of the proximal TJ signalling genes, *CLDN12* and *CTNNA1*, and the RhoGTPase, *RND1*, were compared between CLL B cells and healthy B cells. There was a significant increase in the expression of all three of these genes in CLL cells (**Figure 5.6**).

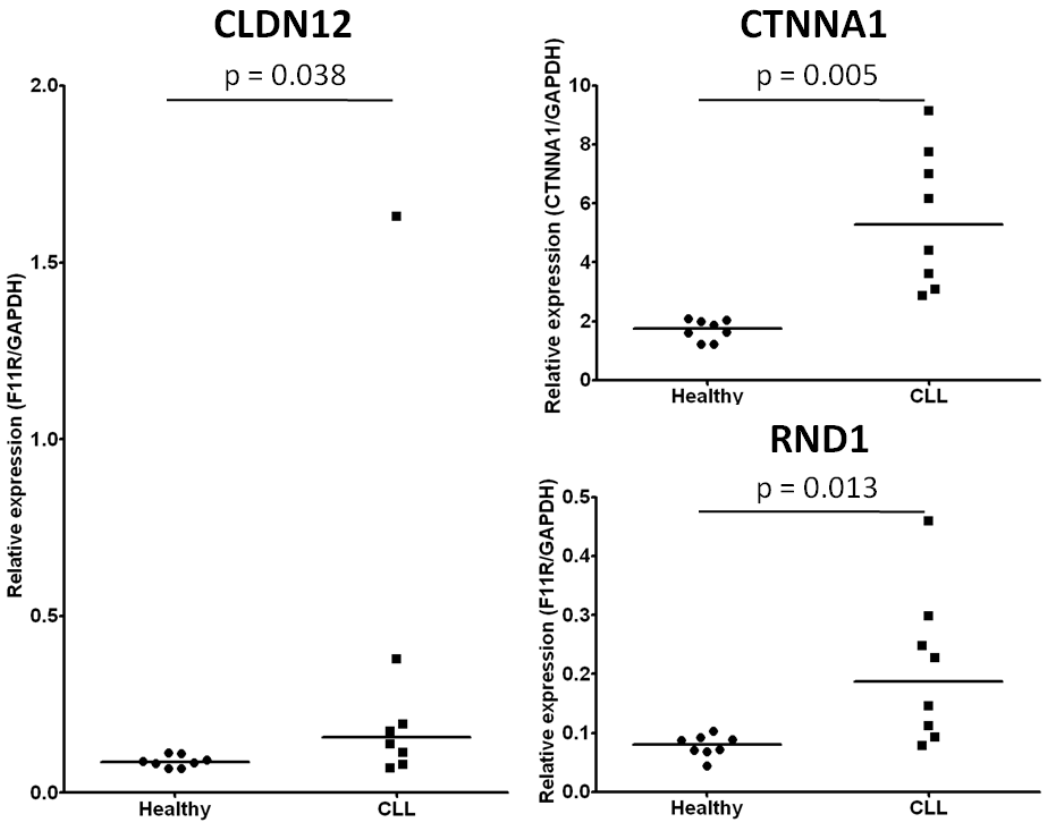



**Figure 5.6 Increased expression of proximal TJ signalling genes, and the RhoGTPase RND1 in CLL cells**

### *5.3.7 Claudin 12, tight junction protein 2, and α-catenin are only very weakly expressed/not expressed in healthy T cells*

Attempts were made to validate alterations in expression of *CLDN12*, *TJP2*, and *CTNNA1* at the protein level using western blotting in healthy T cells. The human embryonic kidney (HEK) cell line, which is known to express these molecules, was used as a positive control. Human T cells did show very low but detectable levels of Claudin-12 protein, but no detectable expression of tight junction protein 2 or α-catenin (**Figure 5.7**). No bands were detectable even after lenalidomide treatment.

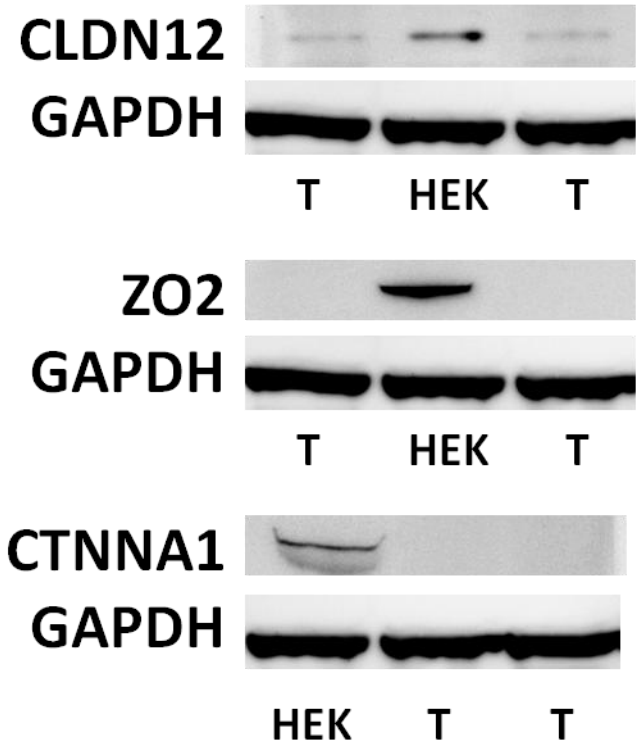



**Figure 5.7 Claudin-12, tight junction protein 2 and α-catenin are only very weakly/not expressed in healthy T cells**

### *5.3.8 Okadaic acid mimics the effect of lenalidomide on T cells*

It was hypothesised that investigation of how the TJ signalling pathway is regulated, would provide novel insights into the underlying mechanism of action of lenalidomide. As discussed, protein phosphatase 2A (PP2A) is a known to be a negative regulator of tight junction signalling and has been implicated in the mechanism of action of lenalidomide in MDS with deletions of 5q. Okadaic acid, a marine toxin that is responsible for the diarrhoeal illness associated with some cases of shellfish poisoning, is a known inhibitor of PP2A. The diarrhoea is thought to be caused by modulation of epithelial tight junctions in the gut, resulting in increased para-cellular permeability, and loss of fluid into the gut lumen. Okadaic is also known to have immunodulatory effects on T cells. It has been shown to enhance the cytotoxicity of $CD8^+$ T cells, to activate co-stimulatory pathways, and to activate AP-1, NF-κB, and TNFα in lymphocytes (Taffs *et al.* 1991; Chuang *et al.* 2000; Kray *et al.* 2005). Therefore the ability of okadaic acid to mimic lenalidomide-enhanced production of IFNγ by anti-CD3 stimulated $CD3^+CD8^+$ T cells was assessed. As shown in **Figure 5.8**, treatment with 10nM okadaic acid was able to mimic the effect of lenalidomide on healthy CD8+ T cells in terms of IFNγ production.

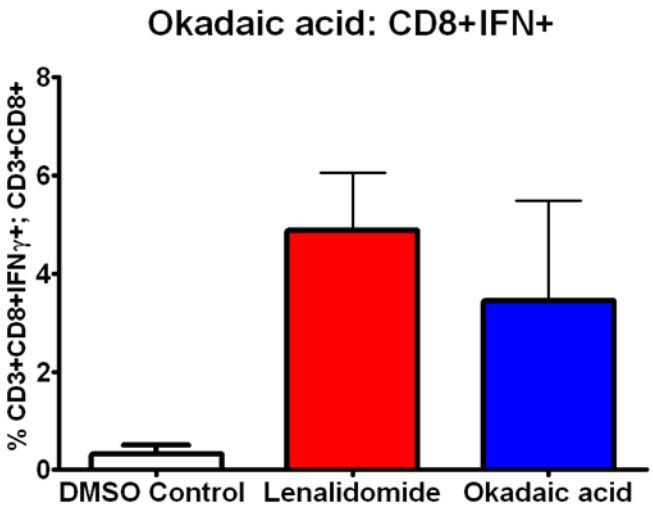



**Figure 5.8 Okadaic acid mimics the ability of lenalidomide to enhanced production of IFNγ by anti-CD3 stimulated $CD3^+CD8^+$ T cells**

### *5.3.9 Okadaic acid mimics the effect of lenalidomide on CLL cells*

The effect of okadaic acid on CLL cells was also assessed. PBMCs from CLL patients were treated with either 10nM okadaic acid or 1μM lenalidomide or vehicle control for 48 hours, before the expression of CD40, CD80, and CD83 was assessed by flow cytometry. Okadaic acid qualitatively mimicked the effect of lenalidomide treatment on CLL cells, with respect to expression of these surface molecules (**Figure 5.9**).

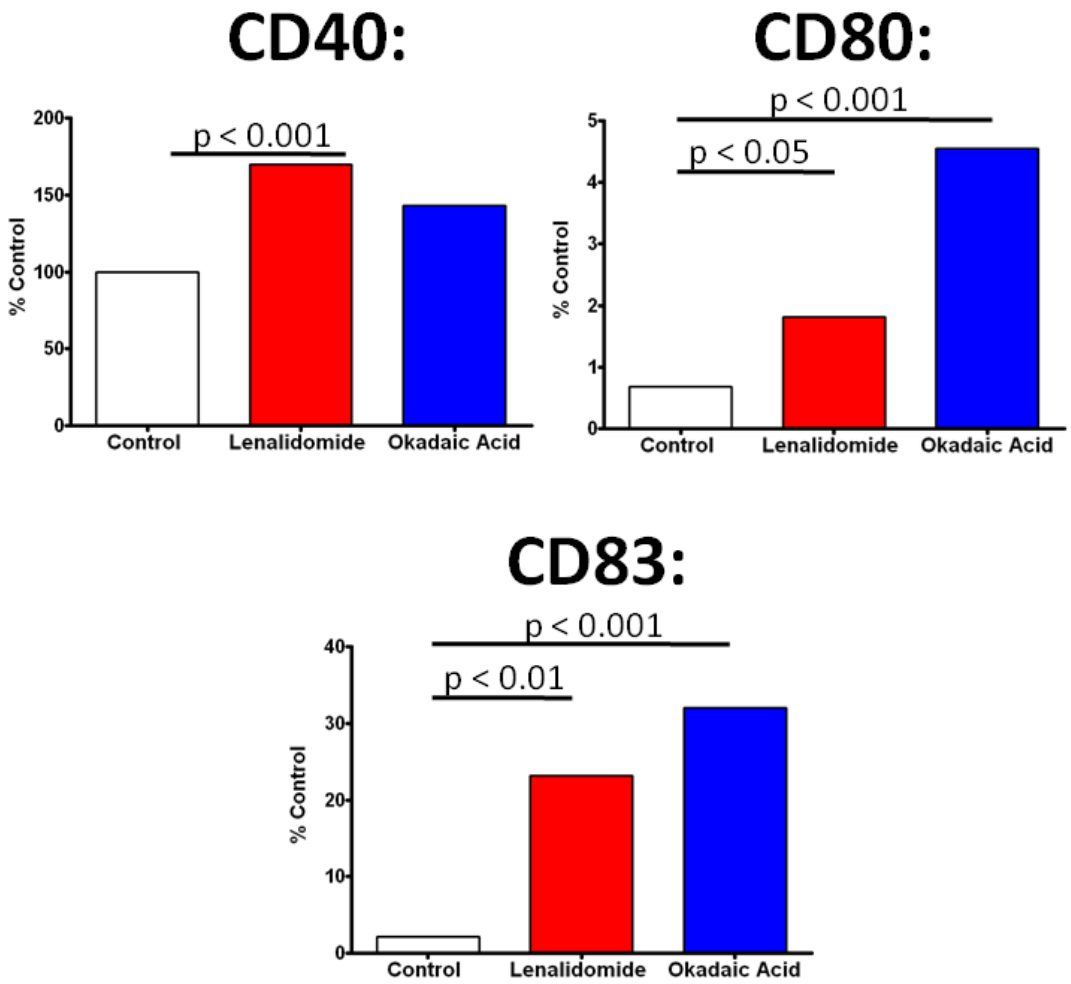



**Figure 5.9 Okadaic acid mimics the upregulation of CD40, CD80 and CD83 induced by lenalidomide treatment of CLL cells.**

## 5.4 Discussion

These results provide evidence that TJ signalling is down-regulated in T cells from CLL patients, particularly in $CD8^+$ T cells and that lenalidomide treatment repairs a defect in this pathway, which results in functional improvements in T-cell activation, immunological synapse formation and cytotoxicity. However, very little is known about the role of TJ signalling in lymphocytes. The junctional adhesion molecules, occludin, and some of the claudins are known to be highly expressed on lymphocyte subsets, but their functional significance remains unclear. Furthermore, investigation of expression of claudin12, α-catenin and TJP2 proteins showed negligible levels of these molecules in healthy primary T cells compared to the HEK293 cell line. It quite possible that upregulation of the TJ pathway by lenalidomide is an epi-phenomenon and little functional significance. However, other molecules downstream in this pathway are known to be crucial in T-cell function, including cdc42, RhoA, Rac1, and TIAM1 (**Figure 5.10**). An activating effect of lenalidomide on RhoA, Rac1, and Cdc42 has been previously demonstrated by our group (Ramsay *et al.* 2012; Ramsay *et al.* 2013). Furthermore, it is reasonable to speculate that components of cell adhesion and motility are widely used and evolutionarily conserved between cell types.

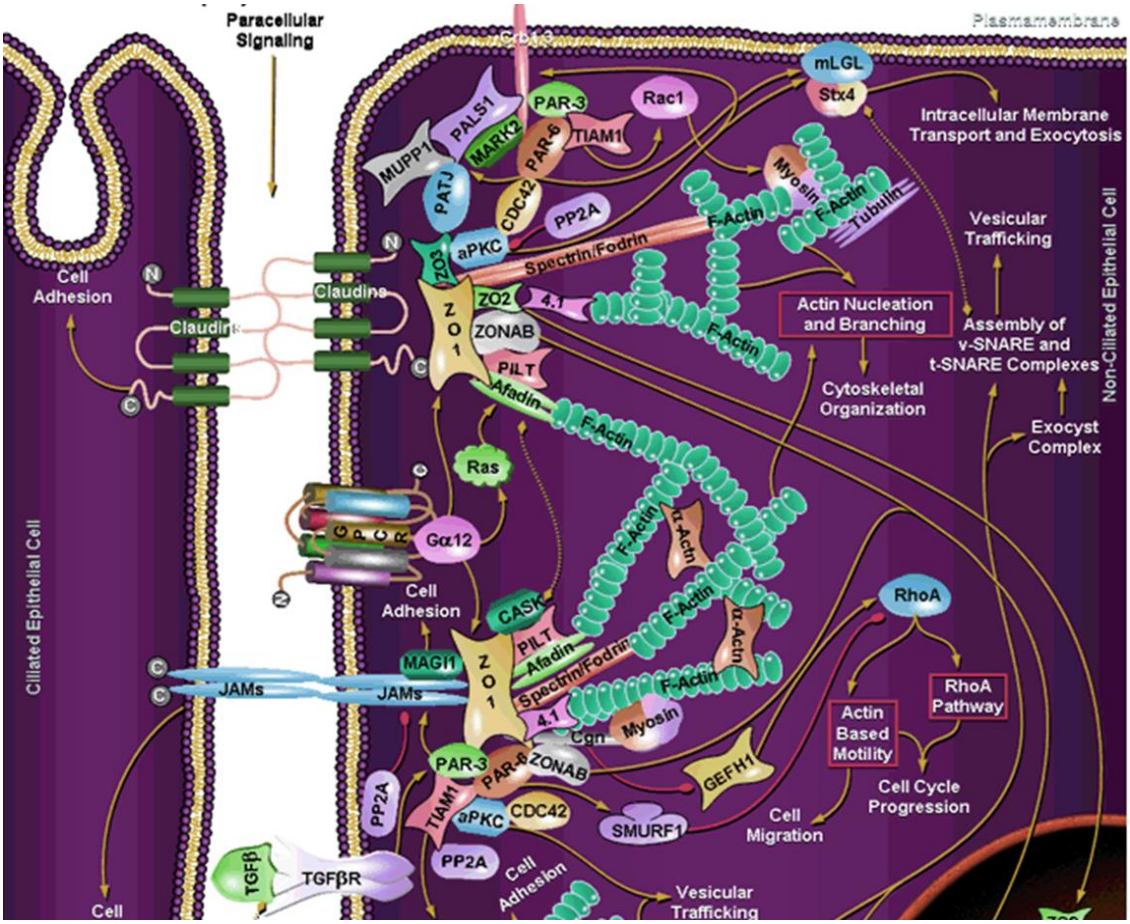



**Figure 5.10 Tight junction signalling pathway (Sabio Biosciences)**

Even if upregulation of components in this pathway do represent an epi-phenomenon in T cells, an understanding of the regulation of TJ signalling should shed new light on the underlying immunomodulatory mechanism of action of lenalidomide. It is reasonable to test the hypothesis that pathways involved in the regulation of TJ signalling may also be implicated in the mechanism of action lenalidomide. One such molecule is the protein phosphatase PP2A, which is known to be a negative regulator of TJ signalling. It could be expected that lenalidomide inhibits PP2A, and indeed evidence from the literature supports this hypothesis. Inhibition of PP2A has been demonstrated to underlie the efficacy of lenalidomide in 5q deleted MDS and to account for the up-regulated of CD154 induced by lenalidomide treatment of CLL (Wei *et al.* 2009; Lapalombella *et al.* 2010). Further support for this has been provided in by the data presented here, which shows that the treatment of CLL cells and T cells with the PP2A inhibitor okadaic acid is able to mimic lenalidomide treatment. While pharmacological manipulation provides some evidence, further work needs to be done. Okadaic acid has several off-target effects, including an ability to inhibit other phosphatases including PP1 and PP2B. A significant amount of effort was directed at optimising a PP2A activity assay (described in section 2.12). Unfortunately due to time constraints this was not completed by the time of thesis submission but will be re-attempted in a period of post-doctoral work. It would also be useful to investigate whether siRNA knockdown of PP2A is able to simulate the effect of lenalidomide and whether the lenalidomide effects in gene expression are antagonised by PP2A activators such as 1,9-di-deoxyforskolin and fingolimod (FTY720).

An aspect of the biology of PP2A that is particularly relevant to T cells is that it mediates the inhibitory effects of CTLA4. CTLA4 is an inhibitory receptor that acts both directly, by decreasing phosphorylation of signalling proteins proximal to the T-cell receptor, and indirectly, by competing with CD28 for CD80/CD86 binding (van der Merwe *et al.* 1997; Lee *et al.* 1998). The cytoplasmic domain of CTLA4 has been shown to associate with PP2A, and CTLA4-mediated inhibition of PI3K/AKT activation has been demonstrated to be sensitive to PP2A inhibition by okadaic acid (Chuang *et al.* 2000; Teft *et al.* 2009). In addition to PD1, CD160, and CD244, many other inhibitory receptors have been implicated in T-cell exhaustion, including CTLA4 (Wherry 2011). While the function of exhausted T-cells can be improved by PD1 blockade alone, the recovery of function is considerably enhanced by simultaneous blockade of PD1 and CTLA4 (Nakamoto *et al.* 2009). This area has considerable

implications for the field of cancer immunotherapy, given the successes reported with CTLA4 blockade in malignant melanoma, both with and without simultaneous blockade of PD1 (Hodi *et al.* 2010; Wolchok *et al.* 2013). It remains unclear whether the CTLA4-PP2A axis plays a role in the immune defect in CLL. An early report showed no demonstrable increase in the expression of CTLA4 on T cells from CLL patients (Scrivener *et al.* 2001). However, other investigators have reported an increase in CTLA4 expression on CLL T cells, along with an expansion of the $CD4^+CD25^+CTLA4^+$ $T_{reg}$ subset (Beyer *et al.* 2005; Motta *et al.* 2005). However, dynamic changes in expression of CTLA4 may be more important, as CLL T cells also show a greater degree of upregulation and prolonged expression of CTLA4 upon T-cell activation, (Frydecka *et al.* 2004). Therefore, it is possible that lenalidomide-induced inhibition of PP2A may "release" T cells from the inhibitory effect of this receptor.

An unexpected finding was that CLL cells showed up-regulation of TJ signalling when compared with healthy $CD19^+$ B cells. Previous observations by my group had shown that lenalidomide treatment of both CLL cells and autologous T cells was required to repair immunological synapse formation. It may be possible that lenalidomide has differential effects on the CLL cells as compared to T cells. One interpretation is that the primary effect of lenalidomide is to repair a cytoskeletal defect in the T cells, but to down-regulate the expression of inhibitory receptors/up-regulate the expression of co-stimulatory ligands on the CLL cells. The activation of TJ signalling in untreated CLL cells may actually allow for increased tissue-homing of the circulating tumour cells, increasing their contact with survival and proliferation signals within lymph nodes. Intriguingly, this upregulation of TJ signalling in CLL cells is consistent with a report showing decreased activity of PP2A in these malignant cells compared to healthy B cells (Yamamoto *et al.* 1999). Indeed inhibition of PP2A is a common event in leukaemia, having also been observed in AML, ALL, and blast crisis CML (Yamamoto *et al.* 1999; Neviani *et al.* 2005; Cristobal *et al.* 2011). Furthermore the activators of PP2A, 1,9-di-deoxyforskolin and fingolimod (FTY720) have shown promising pre-clinical activity for CLL and AML (Liu *et al.* 2008; Cristobal *et al.* 2011). However, it could be speculated that these agents could have potential immuno-suppressive side effects: indeed fingolimod has been used in the treatment of relapsing forms of multiple sclerosis, where it sequester lymphocytes in lymph nodes, preventing them from moving to the central nervous system to induce auto-immunity (Kappos *et al.* 2006).

A second molecule of significant interest in the regulation of TJ signalling is 5' adenosine monophosphate-activated protein kinase (AMPK). AMPK acts as a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of glucose transporter 4 (GLUT4) and mitochondria. In general, activation of AMPK switches on ATP-generating pathways, while switching off ATP-consuming processes. The activity of AMPK is modulated both by the intracellular AMP:ATP ratio and by upstream kinases including AMPK kinase (AMPKK). AMPKK is a complex of three proteins, STE-related adaptor (STRAD), mouse protein 25 (MO25), and liver kinase B1 (LKB1). Interestingly, LKB1 is a serine-threonine kinase and activates AMPK by phosphorylating it at the threonine-172 residue – a process which is known to be directly antagonised by the serine-threonine phosphatase activity of PP2A (Wu *et al.* 2007). AMPK has been shown to be important in the regulation of epithelial tight junctions. AMPK is activated during calcium-induced TJ assembly and this increase depends on the kinase activity of LKB1 (Zheng and Cantley 2007). Furthermore, activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside facilitates TJ assembly under conditions of normal extracellular calcium concentrations and also initiates TJ formation in the absence of calcium (Zhang *et al.* 2006). Intriguingly, the α1 subunit of AMPK has also been shown to interact directly with cereblon (CRBN), which has recently emerged as the target of IMiDs including lenalidomide (Chang and Stewart 2011; Lee *et al.* 2011). AMPK comprises a heterotrimer of a catalytic α subunit, and two regulatory subunits, β and γ. CRBN binding to the AMPK α1 subunit competes with AMPK γ1 for AMPK complex formation, with this competition resulting in decreased activation of AMPK. Therefore, under normal circumstances, CRBN functions as a negative regulator of AMPK. It is therefore possible that binding of IMiD drugs to CRBN reduces its affinity for the AMPK α1 subunit, leading to enhanced AMPK activation.

AMPK is also known to be important for T-cell function. Quiescent naive and memory T cells have a different metabolic profile to effector cells, as the quiescent T cells only require enough energy to prevent cell atrophy and for survival and migration, whereas effector T cells need to proliferate and to release cytolytic molecules and/or effector cytokines. One model that has been proposed is that effector and memory T-cell metabolism is dominated by two opposing metabolic pathways – PI3K/Akt/mTOR-mediated cellular growth and AMPK/Fatty acid oxidation-mediated cellular

homeostasis (Cui and Kaech 2010)(**Figure 5.11**). It is therefore possible that lenalidomide treatment of T cells results in a complete metabolic re-programming that may have implications for exhausted T cells.

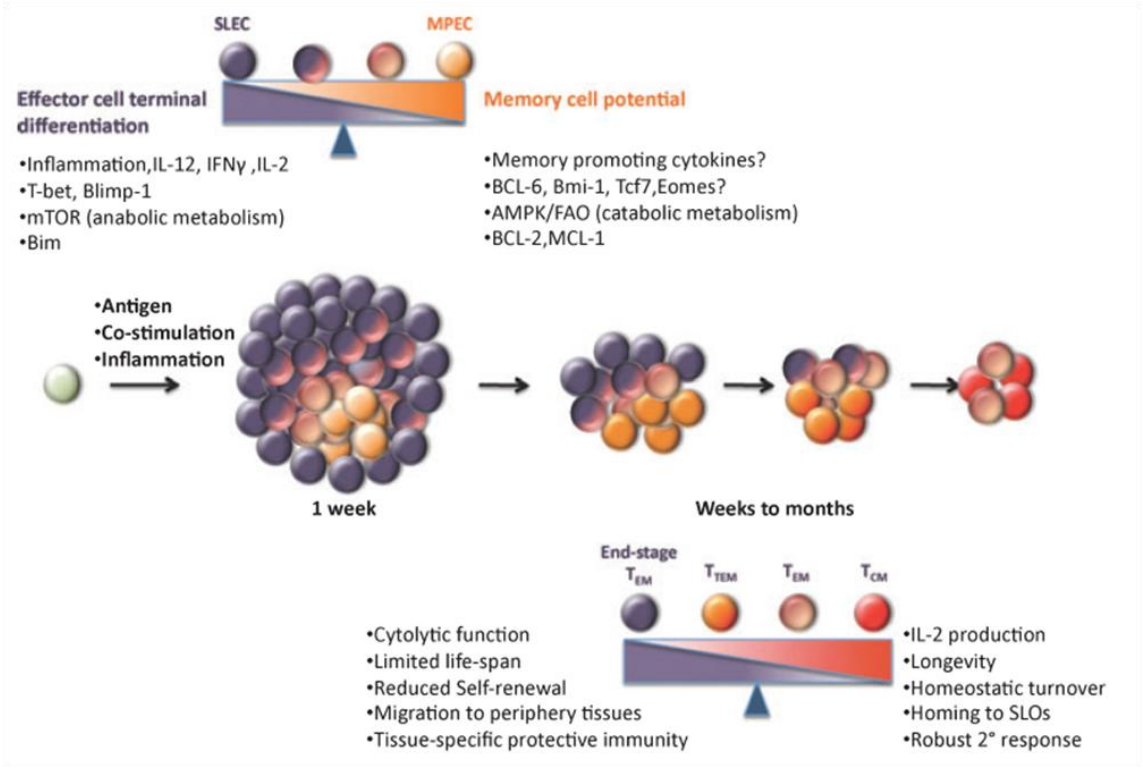



**Figure 5.11 Effector versus memory T cells and their generation**

(Cui and Kaech 2010)

However, this is an emerging area and many aspects remain unclear. Firstly central memory cells are able to generate a strong response to antigen re-encounter, which involves extensive cell proliferation and production of IL2. Furthermore, while LKB1 phosphorylates and activates AMPK in response to increases in cellular AMP:ATP ratios, AMPK can also be activated through a calcium-calmodulin dependent protein kinase pathway in response to TCR triggering, and plays a role in T-cell activation. Indeed it has therefore been proposed that AMPK is important for promoting the conservation and accelerated production of ATP during T-cell activation to regulate energy supplies and stop them becoming depleted (Finlay and Cantrell 2011). Intriguingly, the AMPK activating kinase LKB1 has also been shown to localise to the immunological synapse (Wildenberg *et al.* 2012). In light of these observations, the potential role of AMPK activation in the mechanism of action of lenalidomide will be a topic for further investigation.

# Chapter 6: Discussion

Over the past decade there have been major advances in our understanding of the patho-physiology of CLL, and in the identification of biomarkers that predict the clinical course for individual patients. Over the same period of time, the available therapeutic options have developed dramatically, exemplified by the introduction of combination therapy with purine analogues and monoclonal antibodies, resulting in significant opportunities to induce complete remission (Hallek *et al.* 2010). B-cell receptor signalling inhibitors are showing great promise, but patients are starting to relapse and poor risk subgroups (e.g. deletions or 11q or 17p-) are already showing inferior response rates (Byrd *et al.* 2013). Therefore it does not appear that these agents are a true "cure" for CLL, although their clinical efficacy and better tolerability profile represent a major breakthrough.

Currently, allogeneic HSCT remains the only curative option for CLL. The key to its activity is the graft-versus-leukaemia (GVL) effect, whereby the transplanted haematopoietic stem cells differentiate into effector cells capable of mounting an anti-tumour immune response, which is likely directed at minor host antigenic variations. This effect is known to be primarily T-cell mediated, although it remains unclear whether it is due to improved T-cell function, the presence of allogeneic MHC molecules, or a combination of both (Kolb 2008). One of the major obstacles to the treatment of CLL (in common with other malignancies) is that the tumour cells show a capacity to undergo clonal evolution and develop resistance to any single pharmaceutical treatment. A potential advantage of immunotherapies is that the cellular nature of the anti-cancer effect allows for evolution of the anti-tumour immune response in parallel to the changes in the genetic landscape of the tumour itself. The aim of this thesis was to characterise the nature of the T-cell defect in CLL, and to investigate the mechanism of action of lenalidomide. Lenalidomide is clinically efficacious in CLL, but unlike other agents is not directly toxic to the tumour cells *in vitro*. Instead its efficacy appears to be mediated by immune activation resulting in induction of anti-tumour immune responses, and blockade of pro-tumour factors in the CLL microenvironment. In light of this, it was hypothesised that a deeper understanding of exactly what lenalidomide is "repairing" would lead to further characterisation of the nature of the immune defects. This could then allow for the development of immunomodulatory drugs and therapies that do not have the problematic side effect profile of this agent.

Chronic stimulation is increasingly recognised as being important in the pathogenesis of CLL. Observations regarding the presence of over-represented "stereotyped" BCRs, the importance of prognostic markers associated with BCR-signalling, and the success of inhibitors of BCR-signalling have all indicated that CLL cells derive important survival and proliferation signals from the BCR. It is possible that this represents a property of the BCR itself, leading to antigen-independent cell autonomous signalling, but a wealth of evidence remains supporting a role for antigenic drive (Lanemo Myhrinder *et al.* 2008; Minden *et al.* 2012). In light of this, it was hypothesised that this chronic antigenic drive also affects the T-cell compartment, leading to a state of T-cell exhaustion analogous to that observed in the context of chronic viral infections. This was particularly attractive given the expansion of circulating $CD8^+$ T cells in the peripheral blood of CLL patients, a state which is normally seen in the context of viral infections. Investigation of this did indeed show that circulating T cells from CLL patients exhibited features of T-cell exhaustion, with increased expression of exhaustion markers, a skew towards antigen experienced subsets, an altered transcription factor profile, and functional defects in proliferation and cytotoxicity. The main way in which CLL T cells differed from classically virally exhausted T cells, what that there was no global defect in cytokine production. Exhausted T cells generally lose the ability to produce IL2 initially, with loss of TNFα and IFNγ production at more severe stages of dysfunction. Importantly, these observations have been made in antigen-specific cells in the virally-induced exhaustion, with T-cell function being studied by use of viral peptides. However, it was not possible to do the same investigations in CLL due to the elusive nature of the antigen, necessitating the use of polyclonal stimulation.

There are three main pathways that have been shown to be important in the regulation/promotion of exhausted T cells in addition to chronic antigenic drive. These include inhibitory signalling axis such as the PD1:PDL1 axis, inhibitory T cells subsets such as $T_{regs}$ and inhibitory cytokines such as IL10 (Wherry 2011). My group has focused on the role of inhibitory ligand interactions in CLL. In particular, they have demonstrated that T cells from CLL patients exhibit impaired immunological synapse formation with APCs, and that this defect could be induced in healthy T cells by co-culturing them with CLL cells (Ramsay *et al.* 2008; Ramsay *et al.* 2012). Importantly, subsequent work showed that this effect was mediated by 4 inhibitory ligands that included PDL1 and HVEM, ligands for receptors that are up-regulated on exhausted T cells. Furthermore, $T_{regs}$ are expanded in CLL, are functionally able to suppress

autologous T-cell proliferation, and higher numbers are associated with an impaired prognosis (Beyer *et al.* 2005; Weiss *et al.* 2011). IL10 is also implicated in the pathogenesis of CLL, as patients have higher serum levels of this cytokine compared to controls with higher levels correlating with impaired outcome (Fayad *et al.* 2001). So not only are T cells in CLL phenotypically and functionally exhausted: there is upregulation of these pro-exhaustion pathways as well. However, there are several unanswered questions. The understanding of T-cell exhaustion is incomplete and observations have been primarily made in $CD8^+$ T cells: it remains unclear what the implications are for $CD4^+$ T cells, which are of particular importance in the lymph node microenvironment in CLL. As discussed above, it is also not clear what is driving this chronic stimulation, whether this is antigen, representing infection or autoimmunity or both (molecular mimicry), or whether this due to TAAs on the CLL cells themselves. The significance of the relative preservation of cytokine production is also unclear. It is possible that this is representative of a state distinct from that seen in viral infections, which is a result of chronic stimulation with low affinity self-antigens in contrast to higher affinity viral antigens. It is also possible that this state is induced by the tumour cells as IL2, IFNγ and TNFα have all been shown to be pro-tumour *in vitro* (Yoshizaki *et al.* 1982; Digel *et al.* 1989; Buschle *et al.* 1993). The relative preservation of cytokine production could also be indicative of less exhausted cells, which show a higher capacity for functional restoration. A final question is that T-cell exhaustion is thought to be a chronic process that develops over weeks/months. However, functional defects are inducible in healthy T cells after co-culturing them with CLL cells within 24 hours. My group has demonstrated that inhibitory ligand expression by CLL cells is important for inducing T-cell defects, but it is also likely that other inhibitory factors such as IL10 and $T_{regs}$ play a role in exacerbating any defects caused by chronic antigenic drive.

A major question is whether the "pseudo-exhausted" state of CLL T cells is reversible and whether this represents a potential target for immunotherapeutic manipulation. Much of the pre-clinical work in the context of chronic viral infection has focused on antagonising inhibitory pathways. The PD1/PDL1 axis is a major inhibitory pathway involved in T-cell exhaustion, and has been implicated in the context of several infections including chronic lymphocytic choriomeningitis virus (LCMV) infection in mice, and chronic HIV infection in humans (Barber *et al.* 2006; Day *et al.* 2006). Importantly, these same studies also showed that blocking PD1/PDL1 interactions could restore T-cell effector function, highlighting the functional importance of this axis.

Further work has shown that simultaneous blockade of other inhibitory axes can further enhance T-cell function, such as PD1 blockade in combination with blockade of LAG3, CTLA4 and TIM3 (Blackburn *et al.* 2009; Nakamoto *et al.* 2009; Jin *et al.* 2010). This has led to the concept that partial exhaustion can be reversed relatively easily, but even severely exhausted T cells may respond to combination of modalities aimed at antagonising inhibitory axes, and neutralising the inhibitory effects of $T_{regs}$ and IL10 (Wherry 2011).

Several clinical trials have now shown that anti-PD1 antibodies are effective in chronic viral infections and cancer. In a recently reported clinical trial of PD1 blockade by BMS-936558 in solid cancers, objective responses were seen in 18% of patients with non-small-cell lung cancer, 28% of patients with melanoma, and 27% of patients with renal-cell cancer. Importantly, these responses were also durable with 65% of responses lasting more than a year (Topalian *et al.* 2012). These response rates are very encouraging given the 10-15% "ceiling" of durable tumour response rates seen with trials of other immunotherapeutic approaches in solid cancers over the last 30 years (Ribas 2012). The pre-clinical experience has also led to clinical trials of combination immune checkpoint blockade. Much of the work has been done in melanoma, which appears to be one of the most immuno-sensitive solid cancers. After initial successes with the anti-CTLA4 antibody ipilimumab as a single agent, a recent study has shown improved responses with the combination of ipilimumab in combination with the anti-PD1 antibody nivolumab (Weber *et al.* 2008; Wolchok *et al.* 2013) Furthermore, it has also proven efficacious to target the ligand as well. A phase 1 trial of an anti-PDL1 antibody (BMS-936559) in a variety of advanced solid cancers also showed efficacy, with response rates of 6-17%, again dependent on the cancer type (Brahmer *et al*. 2012). With several inhibitory and activating T-cell receptors available for modulation this area is ripe for investigation (**Figure 6.1)**. It may well be the case that not all approaches are equal, with blockade of different axes being associated with different clinical efficacies and adverse effect profiles. In particular it appears to be safer to block inhibitory pathways rather than target activatory ones, as suggested by the experience with anti-CD28 antibody TGN1412 (Suntharalingam *et al.* 2006). Clinical trials of these agents in haematological malignancies have been notably absent, despite the fact that these cancers are generally more "immunosensitive" with the potential for higher response rates. A single published phase 1 dose-escalation study of the anti-PD1 antibody pidilizumab (CT-011) showed evidence of response in 6 out 18 patients. Of the three

CLL patients that were enrolled, two showed evidence of stable disease, on the second and third dose levels (Berger *et al*. 2008). In light of the pre-clinical data highlighting the significance of the PD1:PDL1 axis in suppressing T-cell function in CLL and the emerging clinical experience in solid cancers, there is strong rationale for clinical assessment of immune checkpoint blockade in this disease.

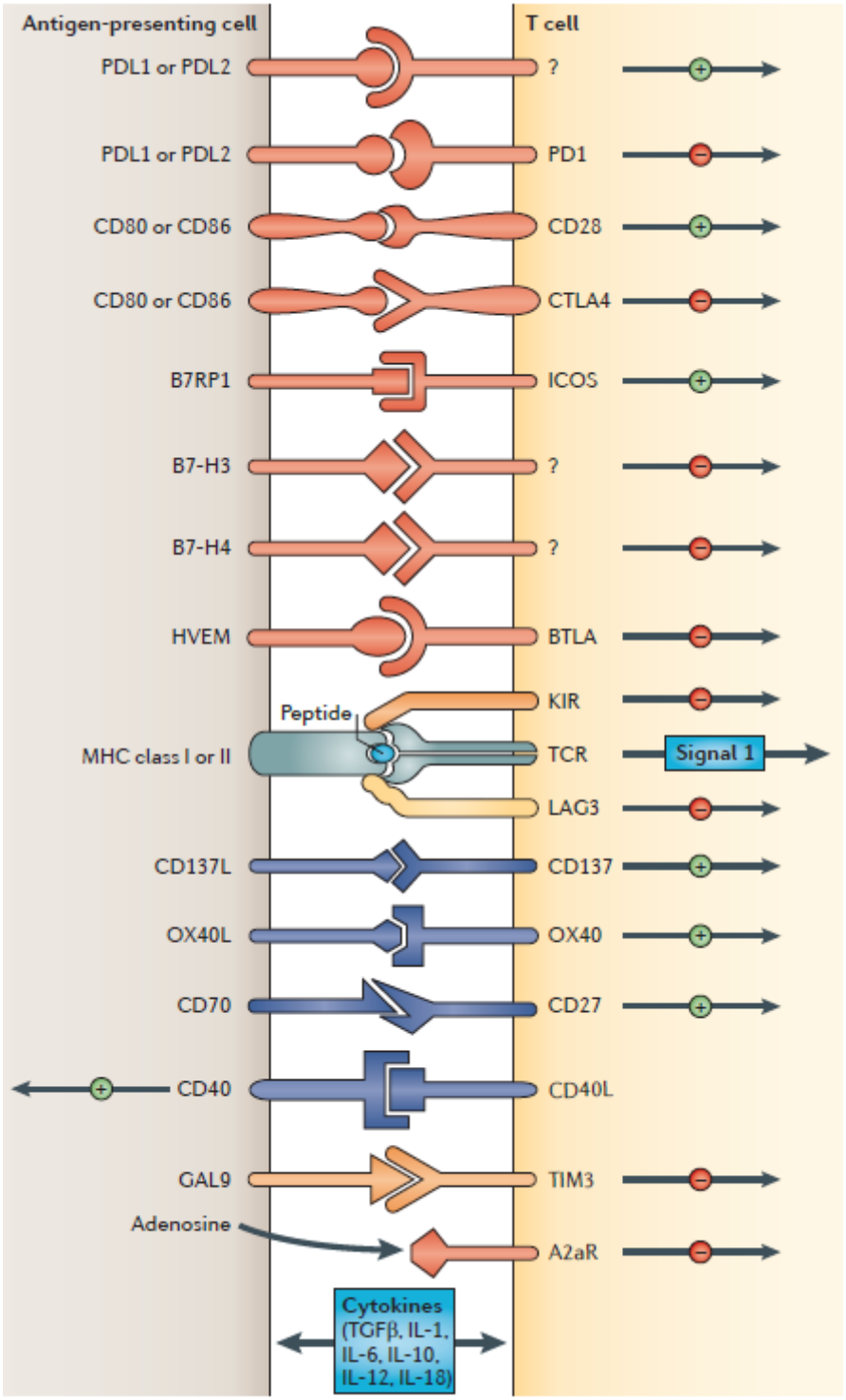



**Figure 6.1 Potential axes for immune checkpoint modulation (Pardoll 2012)**

Given the expression of inhibitory ligands by CLL cells, another way of repairing T cell function may simply be by reducing tumour burden. This is consistent with the experience in allogeneic bone marrow transplantation where patients a have significantly better outcome if transplanted in remission. The immuno-modulatory effect of a reduction in tumour load has previously been difficult to assess, due to the immunosuppressive effect of many standard therapies for CLL, such as chlorambucil, cyclophosphamide and fludarabine. With the development of less immunosuppressive treatments such as the BCR-signalling inhibitors, it will be an important component of future research to establish whether the clinical efficacy of these agents is associated with immune reconstitution. However, it may not be so simple, as these agents could have off-target effects, such as the recently reported inhibitory effect of ibrutinib on IL2-inducible kinase (ITK) (Dubovsky *et al.* 2013). Interestingly, a recent clinical trial that my group was involved with has shown enhanced immune synapse formation after induction therapy with pentostatin, cyclophosphamide, and rituximab, which was further improved by lenalidomide consolidation (Shanafelt *et al.* 2013).

The clinical effectiveness of lenalidomide is of significant interest to the CLL immunotherapy field. As discussed above, it is not directly toxic to CLL cells *in vitro,* but is thought to mediate its effects *in vivo* by immune cell activation and modulation of the microenvironment. It has been shown to have a variety of effects on both T and NK cells including enhanced production of IL2 and IFNγ, enhanced proliferation, and repair of defective immune synapse formation resulting in improved cytotoxicity. Intriguingly, evidence is emerging that lenalidomide also modulates pro-exhaustion pathways. Work by my group has demonstrated that lenalidomide down-regulates both CLL-cell inhibitory ligand expression, and the expression of their receptors on T cells (Ramsay *et al.* 2012). Furthermore, other investigators have observed an effect of lenalidomide on T-cell differentiation, where it is able to inhibit $T_{reg}$ generation instead promoting Th17 differentiation (Idler *et al.* 2009). Therefore, lenalidomide could have a dual effect on exhausted T cells by improving their function directly, but also by inhibiting their formation. The work described in this thesis documents some novel insights into the mechanism of action of lenalidomide, particularly its effect on tight junction signalling, which is potentially involved in lymphocyte motility, transendothelial migration and immunological synapse formation. It has also yielded some further insights in to the molecular pathways targeted by this agent. Inhibition of PP2A is of particular interest given its role in negative co-stimulatory pathways in T cells.

In many respects, T-cell exhaustion is a form of "acquired anergy", with the defects in proliferation and IL2 production representing a failure of co-stimulatory responses. Therefore, lenalidomide treatment may simply repair these defects by providing an over-riding co-stimulatory signal. This also has implications for the CAR T cell field. In a paper that accompanied the publication of a report of the utility of CD19 CAR T cells, phenotyping of the CAR T cells several months post infusion showed them to consist primarily of $CCR7^{-}CD45RA^{+}$ $T_{EMRA}$ cells, with low expression of CD27, CD28 and CD127 and high expression of PD1: an "exhausted" phenotype (Kalos *et al.* 2011). Therefore it is quite possible that the use of the autologous T cells which have been extensively expanded both *in vitro* and *in vivo*, results in cells that are replicatively senescent with incipient loss of function which may result in treatment failure and disease relapse. A particularly interesting part of the most recent developments with CAR T cells is the significance of the co-stimulatory domain used in the CAR construct. It remains unclear as to how domains such as CD28 and CD137 interact with endogenous co-stimulatory elements, and it may be possible that introduction of these elements overcomes defective co-stimulatory pathways in patient T cells and is "protective" against exhaustion.

The recent successes of CAR T cells and lenalidomide have highlighted the "coming of age" of immunotherapy for CLL. These approaches offer the opportunity to provide patients with therapies that reduce rather than exacerbate the immune suppression associated with this disease, allowing for treatment strategies that are viable for all patients, including the elderly and those with co-morbidities. Furthermore, a particularly exciting aspect of these treatments is that they appear to be effective in subgroups of patients who have posed a particular challenge to standard therapy. Recent developments in the fields of immune checkpoint blockade, immunomodulatory drugs, vaccination, and genetically modified T/NK/NKT cells open up the potential for combinations of immunotherapies to finally offer a "cure" for patients with CLL.

# Further work

There were many aspects of this work that require further investigation, but unfortunately time constraints were a major limiting factor. The data described in this thesis does include several novel findings regarding the mechanism of action of lenalidomide that require further work. In particular:

It would be interesting and functionally important to study the effect of lenalidomide on the MDCK cell line, which is a widely used model system to study mammalian tight junction assembly and epithelial polarity regulation.

It would also be important to investigate the role of inhibition of PP2A in lenalidomide's mechanism of action. This could include: further optimisation of a PP2A activity assay to establish whether lenalidomide inhibits this enzyme in T cells; siRNA knockdown of PP2A and pharmacological inhibition of PP2A to investigate whether this mimics lenalidomide's effects on T-cell function and the expression of TJ signalling genes, experimentation with the PP2A agonists 1,9 dideoxyforskolin and fingolimod to analyse whether these agents antagonise the effects of lenalidomide on T-cell function and gene expression.

It would also be logical to investigate the role of AMPK activation in lenalidomide's mechanism of action. This could be directed at assessment of AMPK phosphorylation and also the phosphorylation status of molecules downstream of AMPK such as acetyl-CoA carboxylase. It would also be important to establish whether the effects of lenalidomide described here are mediated by binding to cereblon; this could be tested by seeing if siRNA knockdown of cereblon attenuated these effects.

And finally, it would important to establish if the PP2A-AMPK axis is disturbed in CLL T cells. It could be hypothesised that exhausted T cells and T cells from CLL patients have higher activity of PP2A and lower activity of AMPK consistent with their shift to terminally differentiated subsets.

# Appendices

## Appendix A: Conjugated antibodies for flow cytometry

| Antibody | Clone | Isotype | Company | Catalog Number |
|---|---|---|---|---|
| CD3 FITC | UCHT1 | Mouse $IgG_1$, κ | BD Biosciences | 555332 |
| CD3 PE | UCHT1 | Mouse $IgG_1$, κ | BD Biosciences | 555333 |
| CD3 APC | UCHT1 | Mouse $IgG_1$, κ | BD Biosciences | 555335 |
| CD3 eFluor450 | UCHT1 | Mouse $IgG_1$, κ | eBioscience | 48-0038 |
| CD3 PE-Cy7 | UCHT1 | Mouse IgG1 κ | eBioscience | 25-0038 |
| CD4 FITC | M-T466 | Mouse $IgG_1$, κ | Miltenyi Biotec | 130-080-501 |
| CD4 PE | RPA-T4 | Mouse $IgG_1$, κ | BD Biosciences | 555347 |
| CD4 APC-Cy7 | SK3 | Mouse $IgG_1$, κ | BD Biosciences | 341115 |
| CD4 PerCP | SK3 | Mouse $IgG_1$, κ | BD Biosciences | 345770 |
| CD4 PE-Cy7 | SK3 | Mouse $IgG_1$, κ | eBioscience | 25-0047 |
| CD4 APC-Cy7 | RPA-T4 | Mouse $IgG_1$, κ | eBioscience | 47-0049 |
| CD5 FITC | BL1a | Mouse $IgG_{2a}$, κ | Beckman Coulter | IM0468U |
| CD5 PerCp-Cy5.5 | L17F12 | Mouse $IgG_{2a}$, κ | eBioscience | 45-0058-42 |
| CD8 FITC | SK1 | Mouse $IgG_1$, κ | BD Biosciences | 345772 |
| CD8 PE | SK1 | Mouse $IgG_1$, κ | BD Biosciences | 345773 |
| CD8 PerCP | SK1 | Mouse $IgG_1$, κ | BD Biosciences | 345774 |
| CD8 PerCP-Cy5.5 | RPA-T8 | Mouse $IgG_1$, κ | eBioscience | 45-0088 |
| CD19 FITC | 4G7 | Mouse $IgG_1$, κ | BD Biosciences | 345776 |
| CD19 PE | J3-119 | Mouse $IgG_1$, κ | Beckman Coulter | IM1285 |
| CD19 AF700 | HIB19 | Mouse $IgG_1$, κ | BD Biosciences | 557921 |
| CD19 APC-Cy7 | HIB19 | Mouse $IgG_1$, κ | eBioscience | 47-0199-42 |
| CD20 FITC | 2H7 | Mouse $IgG_{2b}$, κ | BD Biosciences | 555622 |
| CD23 PE | M-L233 | Mouse $IgG_1$, κ | BD Biosciences | 555711 |
| CD28 PE | CD28.2 | Mouse $IgG_1$, κ | Beckman Coulter | IM2071U |
| CD28 FITC | CD28.2 | Mouse $IgG_1$, κ | eBioscience | 11-0289 |
| CD40 FITC | 5C3 | Mouse $IgG_1$, κ | BD Biosciences | 555588 |
| CD40 PE-Cy7 | 5C3 | Mouse $IgG_1$, κ | BD Biosciences | 561215 |
| CD45RA FITC | HI100 | Mouse $IgG_1$, κ | BD Biosciences | 555488 |
| CD45RA PE | HI100 | Mouse $IgG_1$, κ | eBioscience | 12-0458 |
| CD54 APC | HA58 | Mouse $IgG_1$, κ | eBioscience | 17-0549-42 |
| CD56 PE | B159 | Mouse $IgG_1$, κ | BD Biosciences | 555516 |
| CD56 APC | B159 | Mouse $IgG_1$, κ | BD Biosciences | 555518 |
| CD58 FITC | 1C3 | Mouse $IgG_{2a}$, κ | BD Biosciences | 555920 |
| CD59 FITC | OV9A2 | Mouse $IgG_1$, κ | eBioscience | 11-0596-42 |
| CD62L FITC | DREG-56 | Mouse $IgG_1$, κ | eBioscience | 11-0629 |
| CD80 FITC | L307.4 | Mouse $IgG_1$, κ | BD Biosciences | 557226 |
| CD80 PE-Cy7 | L307.4 | Mouse $IgG_1$, κ | BD Biosciences | 561135 |
| CD83 PE | HB15e | Mouse $IgG_1$, κ | BD Biosciences | 556855 |
| CD86 FITC | 2331(FUN-1) | Mouse $IgG_1$, κ | BD Biosciences | 555657 |
| CD86-FITC | 2331(FUN-1) | Mouse $IgG_1$, κ | BD Biosciences | 555657 |

| Antibody | Clone | Isotype | Company | Catalog Number |
|---|---|---|---|---|
| CD95 APC | DX2 | Mouse $IgG_1$, κ | BD Biosciences | 558814 |
| CD107a AF647 | eBioH4A3 | Mouse $IgG_1$, κ | eBioscience | 51-1079 |
| CD122 PE | Mik-b2 | Mouse $IgG_{2a}$, κ | BD Biosciences | 554522 |
| CD127 FITC | eBioRDR5 | Mouse $IgG_1$, κ | eBioscience | 11-1278 |
| CD134 FITC | ACT-35 | Mouse $IgG_1$, κ | eBioscience | 11-1374 |
| CD152 PE | eBio20A | Mouse $IgG_{2a}$, κ | eBioscience | 12-1528 |
| CD160 AF647 | BY55 | Mouse IgM | eBioscience | 51-1609 |
| CD197 PE | 3D12 | Rat $IgG_{2a}$, κ | eBioscience | 12-1979 |
| CD197 APC | 3D12 | Rat $IgG_{2a}$, κ | eBioscience | 17-1979 |
| CD223 APC | Not given | Goat IgG | R&D Systems | FAB2319A |
| CD223 ATTO647N | 17B4 | Mouse $IgG_1$, κ | ENZO Life Sciences | ALX-804-806TS |
| CD244 PE | C1.7 | Mouse $IgG_1$, κ | eBioscience | 12-5838-73 |
| CD252 PE | IK-1 | Mouse $IgG_1$, κ | BD Biosciences | 558164 |
| CD256 PE | T3-6 | Mouse $IgG_{2a}$, κ | Biolegend | 318506 |
| CD262 PE | DJR2-4 | Mouse $IgG_1$, κ | eBioscience | 12-9908-xx |
| CD264 PE | TRAIL-R4-01 | Mouse $IgG_1$, κ | ExBio | 1P-519-C100 |
| CD274 PE-Cy7 | MIH1 | Mouse $IgG_1$, κ | BD Biosciences | 558017 |
| CD279 FITC | MIH4 | Mouse $IgG_1$, κ | BD Biosciences | 557860 |
| CD279 PE | MIH5 | Mouse $IgG_1$, κ | BD Biosciences | 557946 |
| CD279 APC | MIH4 | Mouse $IgG_1$, κ | BD Biosciences | 558694 |
| TIM3 APC | F38-2E2 | Mouse $IgG_1$, κ | eBioscience | 17-3109-42 |
| | | | | |
| **Transcription factors** | | | | |
| BLIMP1 PE | C-21 | Goat IgG | Santa Cruz | 13206 |
| EOMES AF647 | WD1928 | Mouse $IgG_1$, κ | eBioscience | 51-4877-41 |
| TBET PE | eBio4B10 | Mouse $IgG_2$, κ | eBioscience | 12-5825 |
| | | | | |
| **Cytokines** | | | | |
| IFNγ FITC | 4S.B3 | Mouse $IgG_1$, κ | eBioscience | 11-7319 |
| TNFα FITC | MAb11 | Mouse $IgG_1$, κ | BD Biosciences | 554512 |
| IL2 PE | MQ1-17H12 | Rat $IgG_{2a}$, κ | BD Biosciences | 554566 |
| IL4 PE | 8D4-8 | Mouse $IgG_1$, κ | BD Biosciences | 554516 |
| IL4 APC | 8D4-8 | Mouse $IgG_1$, κ | eBioscience | 17-7049 |
| | | | | |
| **Isotype controls** | | | | |
| $IgG_1$, κ FITC | MOPC-21 | Mouse $IgG_1$, κ | BD Biosciences | 555909 |
| $IgG_1$, κ PE | MOPC-21 | Mouse $IgG_1$, κ | BD Biosciences | 559320 |
| $IgG_1$, κ APC | MOPC-21 | Mouse $IgG_1$, κ | BD Biosciences | 555751 |
| $IgG_1$, κ PE-Cy7 | MOPC-21 | Mouse $IgG_1$, κ | BD Biosciences | 557646 |
| $IgG_{2a}$, κ FITC | G155-178 | Mouse $IgG_{2a}$, κ | BD Biosciences | 555573 |
| $IgG_{2a}$, κ PE | G155-178 | Mouse $IgG_{2a}$, κ | BD Biosciences | 559319 |
| $IgG_{2b}$, κ FITC | 27-35 | Mouse $IgG_{2b}$, κ | BD Biosciences | 555742 |

## Appendix B: Genes dysregulated in CLL cells

### Genes ≥ 8 fold up-regulated in CLL cells compared with healthy CD19$^+$ B cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 202709_at | FMOD | fibromodulin | 8.61 | 1.01E-14 |
| 229103_at | WNT3 | wingless-type MMTV integration site family, member 3 | 7.63 | 3.37E-14 |
| 236341_at | CTLA4 | cytotoxic T-lymphocyte-associated protein 4 | 7.17 | 1.55E-08 |
| 1562587_at | CLNK | cytokine-dependent hematopoietic cell linker | 6.90 | 7.87E-15 |
| 231743_at | WNT3 | wingless-type MMTV integration site family, member 3 | 6.80 | 3.46E-11 |
| 204072_s_at | FRY | furry homolog (Drosophila) | 6.28 | 4.64E-11 |
| 219304_s_at | PDGFD | platelet derived growth factor D | 5.88 | 2.07E-14 |
| 217504_at | ABCA6 | ATP-binding cassette, sub-family A (ABC1), member 6 | 5.80 | 4.66E-07 |
| 226408_at | TEAD2 | TEA domain family member 2 | 5.66 | 1.41E-15 |
| 205414_s_at | RICH2 | Rho-type GTPase-activating protein RICH2 | 5.61 | 4.18E-08 |
| 226147_s_at | PIGR | polymeric immunoglobulin receptor | 5.45 | 6.02E-13 |
| 209598_at | PNMA2 | paraneoplastic antigen MA2 | 5.43 | 2.93E-04 |
| 215100_at | C6orf105 | chromosome 6 open reading frame 105 | 5.27 | 6.41E-11 |
| 221455_s_at | WNT3 | wingless-type MMTV integration site family, member 3 | 5.06 | 1.93E-09 |
| 221558_s_at | LEF1 | lymphoid enhancer-binding factor 1 | 4.95 | 5.88E-13 |
| 1570239_a_at | CLNK | cytokine-dependent hematopoietic cell linker | 4.90 | 2.05E-14 |
| 229070_at | C6orf105 | chromosome 6 open reading frame 105 | 4.88 | 4.80E-11 |
| 1554966_a_at | FILIP1L | filamin A interacting protein 1-like | 4.77 | 1.26E-07 |
| 230551_at | KSR2 | kinase suppressor of ras 2 | 4.72 | 3.81E-07 |
| 221078_s_at | CCDC88A | coiled-coil domain containing 88A | 4.72 | 6.61E-14 |
| 201525_at | APOD | apolipoprotein D | 4.62 | 1.80E-07 |
| 229552_at | LOC283454 | hypothetical protein LOC283454 | 4.62 | 2.12E-07 |
| 210948_s_at | LEF1 | lymphoid enhancer-binding factor 1 | 4.59 | 4.28E-10 |
| 210051_at | RAPGEF3 | Rap guanine nucleotide exchange factor (GEF) 3 | 4.58 | 1.11E-13 |
| 225354_s_at | SH3BGRL2 | SH3 domain binding glutamic acid-rich protein like 2 | 4.56 | 7.06E-03 |
| 213714_at | CACNB2 | calcium channel, voltage-dependent, beta 2 subunit | 4.55 | 1.95E-03 |
| 226485_at | VSIG10 | V-set and immunoglobulin domain containing 10 | 4.52 | 5.13E-06 |
| 204135_at | FILIP1L | filamin A interacting protein 1-like | 4.51 | 3.45E-08 |
| 234362_s_at | CTLA4 | cytotoxic T-lymphocyte-associated protein 4 | 4.43 | 1.20E-05 |
| 204731_at | TGFBR3 | transforming growth factor, beta receptor III | 4.36 | 1.95E-05 |
| 213419_at | APBB2 | amyloid beta (A4) precursor protein-binding, B2 | 4.29 | 2.78E-09 |
| 204724_s_at | COL9A3 | collagen, type IX, alpha 3 | 4.23 | 5.81E-07 |
| 206865_at | HRK | harakiri, BCL2 interacting protein | 4.20 | 4.38E-06 |
| 210258_at | RGS13 | regulator of G-protein signaling 13 | 4.15 | 3.59E-02 |
| 239185_at | ABCA9 | ATP-binding cassette, sub-family A (ABC1), member 9 | 4.15 | 2.15E-06 |
| 205805_s_at | ROR1 | receptor tyrosine kinase-like orphan receptor 1 | 4.13 | 2.79E-08 |
| 1553708_at | MGC16075 | hypothetical protein MGC16075 | 4.11 | 4.52E-03 |
| 234440_at | TRD@ | T cell receptor delta locus | 4.08 | 4.85E-10 |
| 231794_at | CTLA4 | cytotoxic T-lymphocyte-associated protein 4 | 4.07 | 3.17E-04 |
| 232821_at | GTSF1L | gametocyte specific factor 1-like | 4.07 | 7.54E-04 |
| 226625_at | TGFBR3 | transforming growth factor, beta receptor III | 4.06 | 4.84E-05 |
| 231472_at | FBXO15 | F-box protein 15 | 3.97 | 6.44E-07 |
| 219654_at | PTPLA | protein tyrosine phosphatase-like, member A | 3.95 | 2.45E-05 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 225045_at | CCDC88A | coiled-coil domain containing 88A | 3.94 | 6.14E-14 |
| 229659_s_at | PIGR | polymeric immunoglobulin receptor | 3.86 | 2.42E-11 |
| 239233_at | CCDC88A | coiled-coil domain containing 88A | 3.83 | 6.15E-12 |
| 225998_at | GAB1 | GRB2-associated binding protein 1 | 3.77 | 8.05E-12 |
| 212985_at | APBB2 | amyloid beta (A4) precursor protein-binding, B2 | 3.74 | 8.46E-10 |
| 221331_x_at | CTLA4 | cytotoxic T-lymphocyte-associated protein 4 | 3.68 | 8.26E-05 |
| 227530_at | AKAP12 | A kinase (PRKA) anchor protein 12 | 3.67 | 6.06E-03 |
| 206864_s_at | HRK | harakiri, BCL2 interacting protein | 3.66 | 8.49E-07 |
| 219387_at | CCDC88A | coiled-coil domain containing 88A | 3.64 | 1.19E-14 |
| 215232_at | RICH2 | Rho-type GTPase-activating protein RICH2 | 3.64 | 5.89E-06 |
| 208195_at | TTN | titin | 3.60 | 2.45E-05 |
| 216748_at | PYHIN1 | pyrin and HIN domain family, member 1 | 3.57 | 3.33E-06 |
| 230489_at | CD5 | CD5 molecule | 3.55 | 1.31E-07 |
| 219578_s_at | CPEB1 | cytoplasmic polyadenylation element binding protein 1 | 3.52 | 2.30E-04 |
| 226002_at | GAB1 | GRB2-associated binding protein 1 | 3.50 | 1.46E-11 |
| 221337_s_at | ADAM29 | ADAM metallopeptidase domain 29 | 3.49 | 1.42E-02 |
| 210550_s_at | RASGRF1 | Ras protein-specific guanine nucleotide-releasing factor 1 | 3.48 | 2.54E-11 |
| 242344_at | GABRB2 | gamma-aminobutyric acid (GABA) A receptor, beta 2 | 3.47 | 6.28E-03 |
| 232060_at | ROR1 | receptor tyrosine kinase-like orphan receptor 1 | 3.45 | 1.94E-06 |
| 220193_at | C1orf113 | chromosome 1 open reading frame 113 | 3.40 | 4.41E-11 |
| 212135_s_at | ATP2B4 | ATPase, Ca++ transporting, plasma membrane 4 | 3.39 | 6.61E-09 |
| 242541_at | ABCA9 | ATP-binding cassette, sub-family A (ABC1), member 9 | 3.39 | 4.90E-05 |
| 229234_at | ZC3H12B | zinc finger CCCH-type containing 12B | 3.36 | 4.05E-10 |
| 229114_at | GAB1 | GRB2-associated binding protein 1 | 3.34 | 1.53E-10 |
| 218872_at | TESC | tescalcin | 3.31 | 1.62E-08 |
| 47553_at | DFNB31 | deafness, autosomal recessive 31 | 3.29 | 1.08E-07 |
| 238462_at | UBASH3B | ubiquitin associated and SH3 domain containing, B | 3.29 | 1.82E-03 |
| 1552552_s_at | CLEC4C | C-type lectin domain family 4, member C | 3.28 | 1.30E-03 |
| 215146_s_at | TTC28 | tetratricopeptide repeat domain 28 | 3.28 | 1.49E-05 |
| 212970_at | APBB2 | amyloid beta precursor protein-binding, B2 | 3.27 | 1.37E-09 |
| 201508_at | IGFBP4 | insulin-like growth factor binding protein 4 | 3.26 | 3.65E-06 |
| 203231_s_at | ATXN1 | ataxin 1 | 3.23 | 1.71E-06 |
| 225806_at | JUB | jub, ajuba homolog (Xenopus laevis) | 3.21 | 1.84E-07 |
| 220784_s_at | UTS2 | urotensin 2 | 3.20 | 1.49E-02 |
| 212838_at | DNMBP | dynamin binding protein | 3.19 | 2.74E-13 |
| 210517_s_at | AKAP12 | A kinase (PRKA) anchor protein 12 | 3.18 | 4.55E-02 |
| 238323_at | TEAD2 | TEA domain family member 2 | 3.18 | 9.34E-09 |
| 1558345_a_at | LOC439911 | hypothetical gene supported by NM_194304 | 3.18 | 6.73E-10 |
| 232820_s_at | GTSF1L | gametocyte specific factor 1-like | 3.15 | 3.35E-03 |
| 212906_at | GRAMD1B | GRAM domain containing 1B | 3.13 | 2.18E-04 |
| 228737_at | TOX2 | TOX high mobility group box family member 2 | 3.13 | 6.77E-06 |
| 200998_s_at | CKAP4 | cytoskeleton-associated protein 4 | 3.12 | 4.88E-09 |
| 1555687_a_at | CLEC4C | C-type lectin domain family 4, member C | 3.12 | 1.03E-03 |
| 225531_at | CABLES1 | Cdk5 and Abl enzyme substrate 1 | 3.09 | 9.40E-05 |
| 206049_at | SELP | selectin P (CD62) | 3.06 | 4.28E-04 |
| 237411_at | ADAMTS6 | ADAM metallopeptidase with thrombospondin motif, 6 | 3.05 | 1.34E-08 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 238587_at | UBASH3B | ubiquitin associated and SH3 domain containing, B | 3.04 | 1.73E-03 |
| 204011_at | SPRY2 | sprouty homolog 2 (Drosophila) | 3.04 | 1.12E-04 |
| 226884_at | LRRN1 | leucine rich repeat neuronal 1 | 3.04 | 7.22E-06 |
| 232533_at | METTL8 | methyltransferase like 8 | 3.02 | 1.19E-06 |
| 236496_at | DEGS2 | degenerative spermatocyte homolog 2, lipid desaturase | 3.02 | 3.36E-09 |
| 243362_s_at | LOC641518 | hypothetical LOC641518 | 3.01 | 3.43E-06 |
| 205489_at | CRYM | crystallin, mu | 3.00 | 1.47E-04 |

## Genes that are ≥ 8 fold down-regulated in CLL cells compared with healthy CD19$^+$ B cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 227646_at | EBF1 | early B-cell factor 1 | 7.76 | 4.12E-07 |
| 207861_at | CCL22 | chemokine (C-C motif) ligand 22 | 7.48 | 1.60E-05 |
| 202589_at | TYMS | thymidylate synthetase | 6.83 | 5.85E-07 |
| 210432_s_at | SCN3A | sodium channel, voltage-gated, type III, alpha subunit | 6.78 | 9.49E-21 |
| 219049_at | CSGALNACT1 | chondroitin sulfate N-acetylgalactosaminyltransferase 1 | 6.76 | 2.07E-11 |
| 205987_at | CD1C | CD1c molecule | 6.59 | 1.93E-06 |
| 214777_at | IGKV4-1 | immunoglobulin kappa variable 4-1 | 6.53 | 5.97E-07 |
| 229487_at | EBF1 | early B-cell factor 1 | 6.42 | 1.33E-08 |
| 203939_at | NT5E | 5'-nucleotidase, ecto (CD73) | 6.42 | 4.50E-07 |
| 217979_at | TSPAN13 | tetraspanin 13 | 6.42 | 6.21E-05 |
| 219648_at | MREG | melanoregulin | 6.39 | 8.22E-08 |
| 209773_s_at | RRM2 | ribonucleotide reductase M2 | 6.29 | 6.05E-08 |
| 232204_at | EBF1 | early B-cell factor 1 | 6.27 | 1.66E-09 |
| 205992_s_at | IL15 | interleukin 15 | 6.20 | 1.97E-07 |
| 202503_s_at | KIAA0101 | KIAA0101 | 5.95 | 5.43E-07 |
| 216491_x_at | IGHM | immunoglobulin heavy constant mu | 5.94 | 9.94E-05 |
| 222449_at | PMEPA1 | prostate transmembrane protein, androgen induced 1 | 5.93 | 6.61E-10 |
| 201890_at | RRM2 | ribonucleotide reductase M2 | 5.91 | 8.89E-07 |
| 239761_at | GCNT1 | glucosaminyl (N-acetyl) transferase 1, core 2 | 5.79 | 6.06E-18 |
| 204416_x_at | APOC1 | apolipoprotein C-I | 5.79 | 1.47E-09 |
| 235048_at | FAM169A | family with sequence similarity 169, member A | 5.75 | 2.74E-13 |
| 243198_at | TEX9 | testis expressed 9 | 5.72 | 1.76E-13 |
| 202269_x_at | GBP1 | guanylate binding protein 1, interferon-inducible, 67kDa | 5.69 | 3.73E-07 |
| 225626_at | PAG1 | phosphoprotein with glycosphingolipid microdomains 1 | 5.69 | 2.38E-08 |
| 204115_at | GNG11 | guanine nucleotide binding protein (G protein), gamma 1 | 5.65 | 3.92E-08 |
| 234764_x_at | IGLV1-44 | immunoglobulin lambda variable 1-44 | 5.65 | 1.55E-05 |
| 222450_at | PMEPA1 | prostate transmembrane protein, androgen induced 1 | 5.62 | 3.16E-13 |
| 225809_at | PARM1 | prostate androgen-regulated mucin-like protein 1 | 5.62 | 1.12E-19 |
| 202345_s_at | FABP5 | fatty acid binding protein 5 (psoriasis-associated) | 5.57 | 9.58E-06 |
| 231577_s_at | GBP1 | guanylate binding protein 1, interferon-inducible, 67kDa | 5.56 | 2.70E-07 |
| 224847_at | CDK6 | cyclin-dependent kinase 6 | 5.53 | 2.43E-15 |
| 218546_at | C1orf115 | chromosome 1 open reading frame 115 | 5.49 | 6.53E-11 |
| 226844_at | MOBKL2B | MOB1, Mps One Binder kinase activator-like 2B (yeast) | 5.49 | 2.78E-16 |
| 231963_at | ANKRD33B | ankyrin repeat domain 33B | 5.48 | 3.32E-11 |
| 202270_at | GBP1 | guanylate binding protein 1, interferon-inducible, 67kDa | 5.48 | 5.13E-07 |
| 204439_at | IFI44L | interferon-induced protein 44-like | 5.45 | 6.61E-04 |
| 225655_at | UHRF1 | ubiquitin-like with PHD and ring finger domains 1 | 5.45 | 4.90E-08 |
| 224851_at | CDK6 | cyclin-dependent kinase 6 | 5.42 | 3.05E-17 |
| 205505_at | GCNT1 | glucosaminyl (N-acetyl) transferase 1, core 2 | 5.41 | 1.09E-15 |
| 225105_at | C12orf75 | chromosome 12 open reading frame 75 | 5.32 | 5.95E-08 |
| 203029_s_at | PTPRN2 | protein tyrosine phosphatase, receptor type, N polypeptide 2 | 5.32 | 6.23E-12 |
| 212813_at | JAM3 | junctional adhesion molecule 3 | 5.28 | 1.09E-15 |
| 219247_s_at | ZDHHC14 | zinc finger, DHHC-type containing 14 | 5.23 | 1.31E-13 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 219355_at | CXorf57 | chromosome X open reading frame 57 | 5.19 | 9.59E-22 |
| 213954_at | FAM169A | family with sequence similarity 169, member A | 5.18 | 9.34E-15 |
| 226702_at | CMPK2 | cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial | 5.18 | 1.81E-04 |
| 228988_at | ZNF711 | zinc finger protein 711 | 5.15 | 5.12E-11 |
| 201417_at | SOX4 | SRY (sex determining region Y)-box 4 | 5.14 | 6.61E-14 |
| 225864_at | FAM84B | family with sequence similarity 84, member B | 5.12 | 5.47E-09 |
| 205139_s_at | UST | uronyl-2-sulfotransferase | 5.08 | 1.57E-08 |
| 209101_at | CTGF | connective tissue growth factor | 5.07 | 6.02E-10 |
| 224435_at | C10orf58 | chromosome 10 open reading frame 58 | 5.05 | 1.18E-06 |
| 223484_at | C15orf48 | chromosome 15 open reading frame 48 | 5.04 | 8.79E-06 |
| 204963_at | SSPN | sarcospan (Kras oncogene-associated gene) | 5.04 | 2.28E-09 |
| 227354_at | PAG1 | phosphoprotein with glycosphingolipid microdomains 1 | 5.03 | 2.72E-09 |
| 206385_s_at | ANK3 | ankyrin 3, node of Ranvier (ankyrin G) | 5.03 | 1.15E-08 |
| 219424_at | EBI3 | Epstein-Barr virus induced 3 | 5.03 | 1.22E-09 |
| 200606_at | DSP | desmoplakin | 5.02 | 1.36E-06 |
| 225710_at | GNB4 | guanine nucleotide binding protein, beta polypeptide 4 | 5.00 | 2.64E-06 |
| 201719_s_at | EPB41L2 | erythrocyte membrane protein band 4.1-like 2 | 4.99 | 3.04E-06 |
| 217967_s_at | FAM129A | family with sequence similarity 129, member A | 4.95 | 1.01E-06 |
| 201830_s_at | NET1 | neuroepithelial cell transforming 1 | 4.95 | 2.61E-07 |
| 203038_at | PTPRK | protein tyrosine phosphatase, receptor type, K | 4.94 | 4.09E-05 |
| 205884_at | ITGA4 | integrin, alpha 4 (CD49D) | 4.93 | 2.12E-07 |
| 203213_at | CDK1 | cyclin-dependent kinase 1 | 4.93 | 8.74E-10 |
| 201324_at | EMP1 | epithelial membrane protein 1 | 4.93 | 2.06E-06 |
| 201928_at | PKP4 | plakophilin 4 | 4.84 | 5.29E-15 |
| 205128_x_at | PTGS1 | prostaglandin-endoperoxide synthase 1 | 4.83 | 6.52E-13 |
| 212558_at | SPRY1 | sprouty homolog 1, antagonist of FGF signaling | 4.82 | 1.36E-12 |
| 205885_s_at | ITGA4 | integrin, alpha 4 (CD49D) | 4.78 | 3.18E-08 |
| 1554696_s_at | TYMS | thymidylate synthetase | 4.77 | 4.28E-10 |
| 212560_at | SORL1 | sortilin-related receptor, L(DLR class) A repeats-containing | 4.76 | 9.70E-04 |
| 219493_at | SHCBP1 | SHC SH2-domain binding protein 1 | 4.75 | 1.01E-09 |
| 244313_at | CR1 | complement component (3b/4b) receptor 1 | 4.72 | 2.05E-11 |
| 229568_at | MOBKL2B | MOB1, Mps One Binder kinase activator-like 2B (yeast) | 4.71 | 1.76E-18 |
| 213416_at | ITGA4 | integrin, alpha 4 (CD49D) | 4.71 | 8.56E-06 |
| 203214_x_at | CDK1 | cyclin-dependent kinase 1 | 4.71 | 6.02E-10 |
| 210559_s_at | CDK1 | cyclin-dependent kinase 1 | 4.71 | 8.47E-10 |
| 208018_s_at | HCK | hemopoietic cell kinase | 4.68 | 2.79E-07 |
| 202336_s_at | PAM | peptidylglycine alpha-amidating monooxygenase | 4.66 | 2.05E-11 |
| 215813_s_at | PTGS1 | prostaglandin-endoperoxide synthase 1 | 4.65 | 3.06E-12 |
| 212975_at | DENND3 | DENN/MADD domain containing 3 | 4.65 | 1.93E-09 |
| 225662_at | ZAK | sterile alpha motif and leucine zipper containing kinase | 4.64 | 8.43E-05 |
| 221530_s_at | BHLHE41 | basic helix-loop-helix family, member e41 | 4.63 | 2.11E-04 |
| 209395_at | CHI3L1 | chitinase 3-like 1 (cartilage glycoprotein-39) | 4.59 | 7.56E-06 |
| 209276_s_at | GLRX | glutaredoxin (thioltransferase) | 4.59 | 3.24E-08 |
| 211597_s_at | HOPX | HOP homeobox | 4.58 | 2.58E-06 |
| 224428_s_at | CDCA7 | cell division cycle associated 7 | 4.58 | 2.01E-09 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 204533_at | CXCL10 | chemokine (C-X-C motif) ligand 10 | 4.58 | 5.04E-05 |
| 218532_s_at | FAM134B | family with sequence similarity 134, member B | 4.57 | 1.75E-10 |
| 222317_at | PDE3B | phosphodiesterase 3B, cGMP-inhibited | 4.57 | 3.74E-07 |
| 229450_at | IFIT3 | interferon-induced protein with tetratricopeptide repeat 3 | 4.56 | 9.66E-04 |
| 202478_at | TRIB2 | tribbles homolog 2 (Drosophila) | 4.55 | 3.35E-08 |
| 217371_s_at | IL15 | interleukin 15 | 4.50 | 6.25E-07 |
| 225330_at | IGF1R | insulin-like growth factor 1 receptor | 4.50 | 4.22E-05 |
| 204249_s_at | LMO2 | LIM domain only 2 (rhombotin-like 1) | 4.49 | 8.31E-09 |
| 215071_s_at | HIST1H2AC | histone cluster 1, H2ac | 4.48 | 2.62E-07 |
| 201739_at | SGK1 | serum/glucocorticoid regulated kinase 1 | 4.48 | 9.52E-07 |
| 218854_at | DSE | dermatan sulfate epimerase | 4.48 | 3.11E-08 |
| 205775_at | FAM50B | family with sequence similarity 50, member B | 4.44 | 1.93E-12 |
| 213817_at | IRAK3 | interleukin-1 receptor-associated kinase 3 | 4.44 | 8.10E-09 |
| 228155_at | C10orf58 | chromosome 10 open reading frame 58 | 4.44 | 7.36E-08 |
| 226017_at | CMTM7 | CKLF-like MARVEL transmembrane domain contain 7 | 4.42 | 2.37E-06 |
| 212274_at | LPIN1 | lipin 1 | 4.41 | 3.75E-05 |
| 219607_s_at | MS4A4A | membrane-spanning 4-domains, subfamily A, member 4 | 4.40 | 9.20E-07 |
| 228698_at | SOX7 | SRY (sex determining region Y)-box 7 | 4.39 | 2.08E-15 |
| 233261_at | EBF1 | early B-cell factor 1 | 4.39 | 1.21E-09 |
| 225202_at | RHOBTB3 | Rho-related BTB domain containing 3 | 4.39 | 5.74E-10 |
| 204591_at | CHL1 | cell adhesion molecule with homology to L1CAM | 4.38 | 4.42E-11 |
| 201829_at | NET1 | neuroepithelial cell transforming 1 | 4.38 | 9.47E-07 |
| 215017_s_at | FNBP1L | formin binding protein 1-like | 4.37 | 5.86E-08 |
| 222838_at | SLAMF7 | SLAM family member 7 | 4.36 | 6.09E-05 |
| 209189_at | FOS | FBJ murine osteosarcoma viral oncogene homolog | 4.33 | 1.17E-06 |
| 234366_x_at | IGL@ | immunoglobulin lambda locus | 4.32 | 5.12E-09 |
| 211696_x_at | HBB | hemoglobin, beta | 4.31 | 1.84E-03 |
| 230509_at | SNX22 | sorting nexin 22 | 4.31 | 1.01E-09 |
| 227361_at | HS3ST3B1 | heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 | 4.30 | 1.08E-08 |
| 217966_s_at | FAM129A | family with sequence similarity 129, member A | 4.29 | 4.71E-09 |
| 235175_at | GBP4 | guanylate binding protein 4 | 4.28 | 3.63E-06 |
| 1553994_at | NT5E | 5'-nucleotidase, ecto (CD73) | 4.27 | 2.59E-07 |
| 203509_at | SORL1 | sortilin-related receptor, L, A repeats-containing | 4.26 | 6.27E-04 |
| 226932_at | SSPN | sarcospan (Kras oncogene-associated gene) | 4.25 | 2.72E-12 |
| 224996_at | ASPH | aspartate beta-hydroxylase | 4.24 | 5.74E-15 |
| 242625_at | RSAD2 | radical S-adenosyl methionine domain containing 2 | 4.24 | 2.41E-04 |
| 212124_at | ZMIZ1 | zinc finger, MIZ-type containing 1 | 4.23 | 2.25E-06 |
| 213624_at | SMPDL3A | sphingomyelin phosphodiesterase, acid-like 3A | 4.22 | 2.44E-08 |
| 223503_at | TMEM163 | transmembrane protein 163 | 4.22 | 1.09E-15 |
| 204491_at | PDE4D | phosphodiesterase 4D, cAMP-specific (Drosophila) | 4.22 | 4.54E-11 |
| 218585_s_at | DTL | denticleless homolog (Drosophila) | 4.21 | 3.35E-08 |
| 203764_at | DLGAP5 | discs, large (Drosophila) homolog-associated protein 5 | 4.20 | 1.12E-07 |
| 1555728_a_at | MS4A4A | membrane-spanning 4-domains, subfamily A, member 4 | 4.20 | 4.15E-07 |
| 228372_at | C10orf128 | chromosome 10 open reading frame 128 | 4.19 | 5.72E-06 |
| 202870_s_at | CDC20 | cell division cycle 20 homolog (S. cerevisiae) | 4.19 | 6.71E-07 |
| 214582_at | PDE3B | phosphodiesterase 3B, cGMP-inhibited | 4.18 | 3.50E-07 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 218793_s_at | SCML1 | sex comb on midleg-like 1 (Drosophila) | 4.18 | 2.81E-06 |
| 213122_at | TSPYL5 | TSPY-like 5 | 4.17 | 1.79E-04 |
| 204493_at | BID | BH3 interacting domain death agonist | 4.16 | 8.93E-07 |
| 211026_s_at | MGLL | monoglyceride lipase | 4.14 | 9.06E-08 |
| 202976_s_at | RHOBTB3 | Rho-related BTB domain containing 3 | 4.13 | 1.25E-09 |
| 230422_at | FPR3 | formyl peptide receptor 3 | 4.13 | 2.45E-05 |
| 214768_x_at | FAM20B | family with sequence similarity 20, member B | 4.13 | 4.20E-02 |
| 212276_at | LPIN1 | lipin 1 | 4.12 | 2.75E-05 |
| 211725_s_at | BID | BH3 interacting domain death agonist | 4.11 | 7.65E-07 |
| 219313_at | GRAMD1C | GRAM domain containing 1C | 4.11 | 1.86E-08 |
| 209642_at | BUB1 | budding uninhibited by benzimidazoles 1 homolog | 4.10 | 9.11E-11 |
| 212820_at | DMXL2 | Dmx-like 2 | 4.09 | 1.14E-06 |
| 225922_at | FNIP2 | folliculin interacting protein 2 | 4.09 | 4.83E-06 |
| 225688_s_at | PHLDB2 | pleckstrin homology-like domain, family B, member 2 | 4.09 | 7.18E-08 |
| 203915_at | CXCL9 | chemokine (C-X-C motif) ligand 9 | 4.09 | 1.26E-06 |
| 202820_at | AHR | aryl hydrocarbon receptor | 4.09 | 5.65E-09 |
| 201565_s_at | ID2 | inhibitor of DNA binding 2 | 4.08 | 7.74E-07 |
| 218662_s_at | NCAPG | non-SMC condensin I complex, subunit G | 4.08 | 5.35E-09 |
| 1554018_at | GPNMB | glycoprotein (transmembrane) nmb | 4.08 | 3.29E-05 |
| 235780_at | PRKACB | protein kinase, cAMP-dependent, catalytic, beta | 4.07 | 1.16E-13 |
| 218284_at | SMAD3 | SMAD family member 3 | 4.06 | 6.30E-05 |
| 223700_at | MND1 | meiotic nuclear divisions 1 homolog (S. cerevisiae) | 4.06 | 2.38E-08 |
| 225665_at | ZAK | sterile alpha motif and leucine zipper containing kinase | 4.03 | 5.36E-05 |
| 209714_s_at | CDKN3 | cyclin-dependent kinase inhibitor 3 | 4.03 | 2.34E-09 |
| 210644_s_at | LAIR1 | leukocyte-associated immunoglobulin-like receptor 1 | 4.03 | 2.00E-05 |
| 209774_x_at | CXCL2 | chemokine (C-X-C motif) ligand 2 | 4.03 | 4.65E-05 |
| 234884_x_at | IGL@ | immunoglobulin lambda locus | 4.01 | 4.75E-06 |
| 209301_at | CA2 | carbonic anhydrase II | 4.01 | 2.78E-12 |
| 201718_s_at | EPB41L2 | erythrocyte membrane protein band 4.1-like 2 | 4.01 | 2.73E-06 |
| 209891_at | SPC25 | NDC80 kinetochore complex component, homolog | 4.00 | 1.08E-08 |
| 204971_at | CSTA | cystatin A (stefin A) | 4.00 | 1.90E-07 |
| 209116_x_at | HBB | hemoglobin, beta | 3.99 | 2.53E-03 |
| 1555756_a_at | CLEC7A | C-type lectin domain family 7, member A | 3.98 | 1.49E-05 |
| 212845_at | SAMD4A | sterile alpha motif domain containing 4A | 3.98 | 7.60E-13 |
| 201291_s_at | TOP2A | topoisomerase (DNA) II alpha 170kDa | 3.97 | 5.44E-07 |
| 225622_at | PAG1 | phosphoprotein with glycosphingolipid microdomains 1 | 3.96 | 3.36E-10 |
| 212923_s_at | C6orf145 | chromosome 6 open reading frame 145 | 3.96 | 9.96E-08 |
| 223922_x_at | MS4A6A | membrane-spanning 4-domains, family A, member 6A | 3.96 | 7.93E-06 |
| 204430_s_at | SLC2A5 | solute carrier family 2, member 5 | 3.95 | 1.40E-05 |
| 235310_at | GCET2 | germinal center expressed transcript 2 | 3.95 | 2.17E-15 |
| 224356_x_at | MS4A6A | membrane-spanning 4-domains, family A, member 6A | 3.95 | 4.80E-06 |
| 204014_at | DUSP4 | dual specificity phosphatase 4 | 3.93 | 2.78E-04 |
| 207900_at | CCL17 | chemokine (C-C motif) ligand 17 | 3.93 | 1.10E-07 |
| 230550_at | MS4A6A | membrane-spanning 4-domains, family A, member 6A | 3.93 | 2.81E-06 |
| 207165_at | HMMR | hyaluronan-mediated motility receptor (RHAMM) | 3.93 | 1.26E-08 |
| 204825_at | MELK | maternal embryonic leucine zipper kinase | 3.92 | 1.36E-07 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 1554519_at | CD80 | CD80 molecule | 3.92 | 2.30E-05 |
| 203922_s_at | CYBB | cytochrome b-245, beta polypeptide | 3.91 | 6.47E-06 |
| 223280_x_at | MS4A6A | membrane-spanning 4-domains, family A, member 6A | 3.91 | 9.77E-06 |
| 221636_s_at | MOSC2 | MOCO sulphurase C-terminal domain containing 2 | 3.90 | 1.84E-08 |
| 229971_at | GPR114 | G protein-coupled receptor 114 | 3.88 | 9.33E-14 |
| 220651_s_at | MCM10 | minichromosome maintenance complex component 10 | 3.88 | 8.24E-08 |
| 220532_s_at | TMEM176B | transmembrane protein 176B | 3.88 | 5.69E-05 |
| 215049_x_at | CD163 | CD163 molecule | 3.88 | 3.70E-05 |
| 1558438_a_at | IGHG1 | immunoglobulin heavy constant gamma 1 | 3.88 | 9.28E-08 |
| 202705_at | CCNB2 | cyclin B2 | 3.87 | 1.87E-08 |
| 211798_x_at | IGLJ3 | immunoglobulin lambda joining 3 | 3.87 | 2.72E-05 |
| 218663_at | NCAPG | non-SMC condensin I complex, subunit G | 3.87 | 1.41E-09 |
| 212592_at | IGJ | immunoglobulin J polypeptide | 3.86 | 6.22E-03 |
| 223344_s_at | MS4A7 | membrane-spanning 4-domains, subfamily A, member 7 | 3.86 | 2.08E-04 |
| 226936_at | C6orf173 | chromosome 6 open reading frame 173 | 3.85 | 4.17E-09 |
| 225673_at | MYADM | myeloid-associated differentiation marker | 3.85 | 3.74E-06 |
| 211881_x_at | IGLJ3 | immunoglobulin lambda joining 3 | 3.84 | 1.17E-05 |
| 227006_at | PPP1R14A | protein phosphatase 1, regulatory (inhibitor) subunit 14A | 3.83 | 5.20E-11 |
| 219148_at | PBK | PDZ binding kinase | 3.82 | 6.99E-09 |
| 205098_at | CCR1 | chemokine (C-C motif) receptor 1 | 3.82 | 5.30E-05 |
| 209135_at | ASPH | aspartate beta-hydroxylase | 3.80 | 1.76E-12 |
| 226456_at | C16orf75 | chromosome 16 open reading frame 75 | 3.80 | 5.74E-10 |
| 204026_s_at | ZWINT | ZW10 interactor | 3.77 | 3.14E-09 |
| 203645_s_at | CD163 | CD163 molecule | 3.77 | 7.74E-05 |
| 211612_s_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 3.77 | 1.41E-05 |
| 232682_at | MREG | melanoregulin | 3.77 | 1.56E-09 |
| 205379_at | CBR3 | carbonyl reductase 3 | 3.76 | 4.06E-11 |
| 217232_x_at | HBB | hemoglobin, beta | 3.75 | 2.70E-03 |
| 1555758_a_at | CDKN3 | cyclin-dependent kinase inhibitor 3 | 3.74 | 5.41E-07 |
| 202742_s_at | PRKACB | protein kinase, cAMP-dependent, catalytic, beta | 3.73 | 1.59E-10 |
| 1553132_a_at | TC2N | tandem C2 domains, nuclear | 3.73 | 1.31E-07 |
| 219666_at | MS4A6A | membrane-spanning 4-domains, family A, member 6A | 3.73 | 4.09E-05 |
| 225897_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 3.72 | 1.66E-04 |
| 217975_at | WBP5 | WW domain binding protein 5 | 3.71 | 3.06E-10 |
| 205681_at | BCL2A1 | BCL2-related protein A1 | 3.71 | 2.67E-04 |
| 224848_at | CDK6 | cyclin-dependent kinase 6 | 3.71 | 6.61E-14 |
| 210904_s_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 3.71 | 2.35E-05 |
| 224358_s_at | MS4A7 | membrane-spanning 4-domains, subfamily A, member 7 | 3.70 | 3.64E-05 |
| 203968_s_at | CDC6 | cell division cycle 6 homolog (S. cerevisiae) | 3.69 | 8.14E-08 |
| 204015_s_at | DUSP4 | dual specificity phosphatase 4 | 3.69 | 6.42E-06 |
| 214453_s_at | IFI44 | interferon-induced protein 44 | 3.69 | 3.04E-03 |
| 242794_at | MAML3 | mastermind-like 3 (Drosophila) | 3.66 | 3.23E-12 |
| 228868_x_at | CDT1 | chromatin licensing and DNA replication factor 1 | 3.66 | 5.29E-09 |
| 214039_s_at | LAPTM4B | lysosomal protein transmembrane 4 beta | 3.65 | 2.94E-03 |
| 219159_s_at | SLAMF7 | SLAM family member 7 | 3.65 | 1.41E-05 |
| 220377_at | FAM30A | family with sequence similarity 30, member A | 3.65 | 2.29E-02 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 201310_s_at | C5orf13 | chromosome 5 open reading frame 13 | 3.65 | 1.09E-06 |
| 202087_s_at | CTSL1 | cathepsin L1 | 3.64 | 7.81E-06 |
| 232231_at | RUNX2 | runt-related transcription factor 2 | 3.63 | 2.27E-07 |
| 206478_at | KIAA0125 | KIAA0125 | 3.63 | 2.72E-02 |
| 201292_at | TOP2A | topoisomerase (DNA) II alpha 170kDa | 3.62 | 3.09E-07 |
| 222680_s_at | DTL | denticleless homolog (Drosophila) | 3.62 | 3.48E-08 |
| 204747_at | IFIT3 | interferon-induced protein with tetratricopeptide repeat 3 | 3.62 | 2.61E-03 |
| 206488_s_at | CD36 | CD36 molecule (thrombospondin receptor) | 3.61 | 9.89E-05 |
| 203418_at | CCNA2 | cyclin A2 | 3.61 | 1.19E-08 |
| 202954_at | UBE2C | ubiquitin-conjugating enzyme E2C | 3.61 | 6.16E-08 |
| 227697_at | SOCS3 | suppressor of cytokine signaling 3 | 3.60 | 4.27E-05 |
| 208820_at | PTK2 | PTK2 protein tyrosine kinase 2 | 3.59 | 4.93E-03 |
| 206214_at | PLA2G7 | phospholipase A2, group VII | 3.59 | 2.05E-05 |
| 233969_at | IGL@ | immunoglobulin lambda locus | 3.58 | 9.43E-07 |
| 1553995_a_at | NT5E | 5'-nucleotidase, ecto (CD73) | 3.58 | 3.10E-09 |
| 201631_s_at | IER3 | immediate early response 3 | 3.58 | 1.71E-06 |
| 206420_at | IGSF6 | immunoglobulin superfamily, member 6 | 3.58 | 7.99E-07 |
| 204112_s_at | HNMT | histamine N-methyltransferase | 3.57 | 1.02E-04 |
| 200648_s_at | GLUL | glutamate-ammonia ligase (glutamine synthetase) | 3.57 | 7.82E-04 |
| 203923_s_at | CYBB | cytochrome b-245, beta polypeptide | 3.57 | 1.87E-04 |
| 215543_s_at | LARGE | like-glycosyltransferase | 3.56 | 4.87E-16 |
| 219386_s_at | SLAMF8 | SLAM family member 8 | 3.55 | 2.82E-06 |
| 225603_s_at | C8orf83 | chromosome 8 open reading frame 83 | 3.55 | 1.17E-04 |
| 204646_at | DPYD | dihydropyrimidine dehydrogenase | 3.54 | 2.35E-03 |
| 205003_at | DOCK4 | dedicator of cytokinesis 4 | 3.54 | 8.94E-06 |
| 201141_at | GPNMB | glycoprotein (transmembrane) nmb | 3.54 | 4.98E-06 |
| 204639_at | ADA | adenosine deaminase | 3.54 | 5.68E-06 |
| 219918_s_at | ASPM | asp homolog, microcephaly associated (Drosophila) | 3.54 | 2.26E-06 |
| 204962_s_at | CENPA | centromere protein A | 3.53 | 2.28E-06 |
| 214595_at | KCNG1 | potassium voltage-gated channel, subfamily G, member 1 | 3.53 | 7.91E-11 |
| 1555745_a_at | LYZ | lysozyme (renal amyloidosis) | 3.52 | 7.03E-04 |
| 208636_at | ACTN1 | actinin, alpha 1 | 3.52 | 2.72E-05 |
| 206039_at | RAB33A | RAB33A, member RAS oncogene family | 3.51 | 2.74E-13 |
| 219527_at | MOSC2 | MOCO sulphurase C-terminal domain containing 2 | 3.51 | 1.80E-07 |
| 203139_at | DAPK1 | death-associated protein kinase 1 | 3.51 | 2.18E-05 |
| 201670_s_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 3.50 | 6.70E-05 |
| 234970_at | TC2N | tandem C2 domains, nuclear | 3.49 | 6.22E-06 |
| 225924_at | FNIP2 | folliculin interacting protein 2 | 3.49 | 6.44E-07 |
| 235574_at | GBP4 | guanylate binding protein 4 | 3.48 | 3.56E-06 |
| 208092_s_at | FAM49A | family with sequence similarity 49, member A | 3.47 | 1.58E-04 |
| 203153_at | IFIT1 | interferon-induced protein with tetratricopeptide repeat 1 | 3.47 | 1.34E-02 |
| 201566_x_at | ID2 | inhibitor of DNA binding 2 | 3.47 | 7.89E-08 |
| 209683_at | FAM49A | family with sequence similarity 49, member A | 3.46 | 5.40E-03 |
| 221524_s_at | RRAGD | Ras-related GTP binding D | 3.46 | 1.36E-07 |
| 226460_at | FNIP2 | folliculin interacting protein 2 | 3.46 | 4.90E-06 |
| 201927_s_at | PKP4 | plakophilin 4 | 3.45 | 2.30E-09 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 204103_at | CCL4 | chemokine (C-C motif) ligand 4 | 3.45 | 7.82E-04 |
| 238669_at | PTGS1 | prostaglandin-endoperoxide synthase 1 | 3.45 | 1.32E-10 |
| 223307_at | CDCA3 | cell division cycle associated 3 | 3.44 | 9.96E-08 |
| 217552_x_at | CR1 | complement component (3b/4b) receptor 1 | 3.43 | 4.74E-13 |
| 206632_s_at | APOBEC3B | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B | 3.43 | 3.29E-07 |
| 221704_s_at | VPS37B | vacuolar protein sorting 37 homolog B (S. cerevisiae) | 3.42 | 6.48E-05 |
| 223159_s_at | NEK6 | NIMA (never in mitosis gene a)-related kinase 6 | 3.42 | 1.01E-06 |
| 202859_x_at | IL8 | interleukin 8 | 3.42 | 7.61E-03 |
| 201325_s_at | EMP1 | epithelial membrane protein 1 | 3.42 | 9.21E-06 |
| 212188_at | KCTD12 | potassium channel tetramerisation domain containing 12 | 3.41 | 9.08E-06 |
| 202741_at | PRKACB | protein kinase, cAMP-dependent, catalytic, beta | 3.41 | 1.80E-08 |
| 227647_at | KCNE3 | potassium voltage-gated channel, Isk-related family, member 3 | 3.40 | 6.09E-06 |
| 213241_at | PLXNC1 | plexin C1 | 3.40 | 1.52E-07 |
| 226611_s_at | CENPV | centromere protein V | 3.40 | 1.99E-05 |
| 224553_s_at | TNFRSF18 | tumour necrosis factor receptor superfamily, member 18 | 3.37 | 1.16E-04 |
| 227449_at | EPHA4 | EPH receptor A4 | 3.36 | 9.26E-08 |
| 202796_at | SYNPO | synaptopodin | 3.36 | 1.66E-09 |
| 203030_s_at | PTPRN2 | protein tyrosine phosphatase, receptor type, N polypeptide 2 | 3.36 | 2.73E-09 |
| 209884_s_at | SLC4A7 | solute carrier family 4, member 7 | 3.36 | 1.63E-03 |
| 1563209_a_at | MACROD2 | MACRO domain containing 2 | 3.36 | 1.04E-04 |
| 210139_s_at | PMP22 | peripheral myelin protein 22 | 3.36 | 4.60E-05 |
| 240890_at | LOC643733 | hypothetical LOC643733 | 3.35 | 5.04E-07 |
| 223343_at | MS4A7 | membrane-spanning 4-domains, subfamily A, member 7 | 3.35 | 5.96E-04 |
| 228766_at | CD36 | CD36 molecule (thrombospondin receptor) | 3.35 | 1.72E-04 |
| 208146_s_at | CPVL | carboxypeptidase, vitellogenic-like | 3.35 | 5.04E-05 |
| 202095_s_at | BIRC5 | baculoviral IAP repeat-containing 5 | 3.35 | 4.05E-05 |
| 213797_at | RSAD2 | radical S-adenosyl methionine domain containing 2 | 3.35 | 4.25E-04 |
| 211429_s_at | SERPINA1 | serpin peptidase inhibitor, clade A, member 1 | 3.34 | 4.90E-05 |
| 209191_at | TUBB6 | tubulin, beta 6 | 3.34 | 3.61E-03 |
| 201785_at | RNASE1 | ribonuclease, RNase A family, 1 (pancreatic) | 3.34 | 8.15E-05 |
| 1569481_s_at | SNX22 | sorting nexin 22 | 3.33 | 4.85E-10 |
| 211794_at | FYB | FYN binding protein (FYB-120/130) | 3.33 | 7.18E-05 |
| 212446_s_at | LASS6 | LAG1 homolog, ceramide synthase 6 | 3.32 | 8.34E-03 |
| 200862_at | DHCR24 | 24-dehydrocholesterol reductase | 3.31 | 1.39E-05 |
| 213002_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 3.31 | 3.73E-06 |
| 206118_at | STAT4 | signal transducer and activator of transcription 4 | 3.31 | 7.31E-05 |
| 229560_at | TLR8 | toll-like receptor 8 | 3.31 | 6.44E-04 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 3.31 | 1.15E-04 |
| 226066_at | MITF | microphthalmia-associated transcription factor | 3.31 | 1.41E-05 |
| 205569_at | LAMP3 | lysosomal-associated membrane protein 3 | 3.30 | 5.56E-05 |
| 218510_x_at | FAM134B | family with sequence similarity 134, member B | 3.28 | 2.80E-12 |
| 227143_s_at | BID | BH3 interacting domain death agonist | 3.28 | 1.36E-06 |
| 206978_at | CCR2 | chemokine (C-C motif) receptor 2 | 3.28 | 2.61E-05 |
| 221210_s_at | NPL | N-acetylneuraminate pyruvate lyase | 3.27 | 1.46E-05 |
| 217875_s_at | PMEPA1 | prostate transmembrane protein, androgen induced 1 | 3.27 | 1.05E-12 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 208949_s_at | LGALS3 | lectin, galactoside-binding, soluble, 3 | 3.27 | 2.34E-06 |
| 209684_at | RIN2 | Ras and Rab interactor 2 | 3.27 | 3.41E-04 |
| 221523_s_at | RRAGD | Ras-related GTP binding D | 3.26 | 2.89E-06 |
| 212192_at | KCTD12 | potassium channel tetramerisation domain containing 12 | 3.26 | 2.26E-05 |
| 205237_at | FCN1 | ficolin (collagen/fibrinogen domain containing) 1 | 3.25 | 2.39E-04 |
| 205692_s_at | CD38 | CD38 molecule | 3.25 | 3.92E-08 |
| 201445_at | CNN3 | calponin 3, acidic | 3.25 | 3.50E-03 |
| 209679_s_at | SMAGP | small cell adhesion glycoprotein | 3.23 | 5.01E-09 |
| 202191_s_at | GAS7 | growth arrest-specific 7 | 3.23 | 1.19E-06 |
| 202551_s_at | CRIM1 | cysteine rich transmembrane BMP regulator 1 | 3.22 | 2.58E-06 |
| 206983_at | CCR6 | chemokine (C-C motif) receptor 6 | 3.22 | 3.28E-04 |
| 201280_s_at | DAB2 | disabled homolog 2, mitogen-responsive phosphoprotein | 3.22 | 1.14E-04 |
| 206392_s_at | RARRES1 | retinoic acid receptor responder (tazarotene induced) 1 | 3.22 | 4.75E-04 |
| 219788_at | PILRA | paired immunoglobin-like type 2 receptor alpha | 3.22 | 1.83E-05 |
| 221872_at | RARRES1 | retinoic acid receptor responder (tazarotene induced) 1 | 3.21 | 1.96E-04 |
| 202479_s_at | TRIB2 | tribbles homolog 2 (Drosophila) | 3.21 | 4.64E-11 |
| 225353_s_at | C1QC | complement component 1, q subcomponent, C chain | 3.20 | 6.30E-07 |
| 218559_s_at | MAFB | v-maf musculoaponeurotic fibrosarcoma oncogene, B | 3.19 | 1.92E-04 |
| 209555_s_at | CD36 | CD36 molecule (thrombospondin receptor) | 3.19 | 1.71E-03 |
| 227486_at | NT5E | 5'-nucleotidase, ecto (CD73) | 3.19 | 1.92E-08 |
| 203474_at | IQGAP2 | IQ motif containing GTPase activating protein 2 | 3.19 | 3.20E-04 |
| 227417_at | MOSC2 | MOCO sulphurase C-terminal domain containing 2 | 3.18 | 7.94E-06 |
| 205099_s_at | CCR1 | chemokine (C-C motif) receptor 1 | 3.17 | 3.69E-05 |
| 205394_at | CHEK1 | CHK1 checkpoint homolog (S. pombe) | 3.17 | 1.16E-07 |
| 221724_s_at | CLEC4A | C-type lectin domain family 4, member A | 3.15 | 1.23E-04 |
| 242136_x_at | MGC70870 | C-terminal binding protein 2 pseudogene | 3.14 | 1.59E-06 |
| 204232_at | FCER1G | Fc fragment of IgE, receptor for; gamma polypeptide | 3.14 | 1.50E-04 |
| 209732_at | CLEC2B | C-type lectin domain family 2, member B | 3.14 | 8.69E-03 |
| 218542_at | CEP55 | centrosomal protein 55kDa | 3.13 | 2.33E-06 |
| 224009_x_at | DHRS9 | dehydrogenase/reductase (SDR family) member 9 | 3.13 | 2.46E-05 |
| 232798_at | DNAJC5B | DnaJ (Hsp40) homolog, subfamily C, member 5 beta | 3.13 | 2.26E-08 |
| 201888_s_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 3.13 | 1.22E-05 |
| 202599_s_at | NRIP1 | nuclear receptor interacting protein 1 | 3.13 | 9.14E-03 |
| 201887_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 3.12 | 4.86E-05 |
| 209709_s_at | HMMR | hyaluronan-mediated motility receptor (RHAMM) | 3.12 | 1.47E-07 |
| 206115_at | EGR3 | early growth response 3 | 3.12 | 1.48E-02 |
| 210448_s_at | P2RX5 | purinergic receptor P2X, ligand-gated ion channel, 5 | 3.11 | 3.60E-05 |
| 238756_at | GAS2L3 | growth arrest-specific 2 like 3 | 3.11 | 2.77E-05 |
| 222670_s_at | MAFB | v-maf musculoaponeurotic fibrosarcoma oncogene, B | 3.11 | 2.49E-04 |
| 219000_s_at | DSCC1 | defective in sister chromatid cohesion 1 homolog | 3.11 | 3.09E-08 |
| 204822_at | TTK | TTK protein kinase | 3.10 | 1.48E-08 |
| 219836_at | ZBED2 | zinc finger, BED-type containing 2 | 3.10 | 1.92E-06 |
| 203936_s_at | MMP9 | matrix metallopeptidase 9 | 3.09 | 3.06E-03 |
| 1554899_s_at | FCER1G | Fc fragment of IgE, receptor for; gamma polypeptide | 3.09 | 1.46E-04 |
| 202600_s_at | NRIP1 | nuclear receptor interacting protein 1 | 3.09 | 1.19E-02 |
| 212364_at | MYO1B | myosin IB | 3.09 | 1.16E-07 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 202975_s_at | RHOBTB3 | Rho-related BTB domain containing 3 | 3.07 | 1.70E-09 |
| 205372_at | PLAG1 | pleiomorphic adenoma gene 1 | 3.07 | 1.25E-08 |
| 202833_s_at | SERPINA1 | serpin peptidase inhibitor, clade A, member 1 | 3.07 | 8.61E-04 |
| 232504_at | LOC285628 | hypothetical protein LOC285628 | 3.07 | 1.58E-04 |
| 218692_at | GOLSYN | Golgi-localized protein | 3.07 | 7.31E-04 |
| 225589_at | SH3RF1 | SH3 domain containing ring finger 1 | 3.06 | 5.29E-10 |
| 1555349_a_at | ITGB2 | integrin, beta 2 | 3.05 | 1.21E-04 |
| 220088_at | C5AR1 | complement component 5a receptor 1 | 3.05 | 1.05E-04 |
| 201669_s_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 3.05 | 1.99E-04 |
| 201743_at | CD14 | CD14 molecule | 3.05 | 1.59E-03 |
| 212526_at | SPG20 | spastic paraplegia 20 (Troyer syndrome) | 3.05 | 3.41E-02 |
| 223204_at | FAM198B | family with sequence similarity 198, member B | 3.04 | 7.26E-04 |
| 223319_at | GPHN | gephyrin | 3.04 | 8.98E-05 |
| 212022_s_at | MKI67 | antigen identified by monoclonal antibody Ki-67 | 3.03 | 7.22E-07 |
| 209264_s_at | TSPAN4 | tetraspanin 4 | 3.03 | 3.75E-05 |
| 205034_at | CCNE2 | cyclin E2 | 3.03 | 1.25E-08 |
| 209959_at | NR4A3 | nuclear receptor subfamily 4, group A, member 3 | 3.02 | 2.20E-03 |
| 226039_at | MGAT4A | mannosyl glycoprotein beta-1,4-N-acetylglucosaminyltransferase A | 3.01 | 1.45E-04 |
| 209933_s_at | CD300A | CD300a molecule | 3.01 | 3.75E-05 |
| 230276_at | FAM49A | family with sequence similarity 49, member A | 3.01 | 6.74E-03 |
| 208158_s_at | OSBPL1A | oxysterol binding protein-like 1A | 3.00 | 8.55E-08 |

## Appendix C: Genes dysregulated in $CD4^+$ T cells from CLL patients

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 1560676_at | SIAH3 | seven in absentia homolog 3 (Drosophila) | 2.207 | 0.012 |
| 225227_at | NA | NA | 1.443 | 0.034 |
| 204014_at | DUSP4 | dual specificity phosphatase 4 | 1.183 | 0.045 |
| 230850_at | NA | NA | 0.865 | 0.044 |
| 235652_at | NA | NA | 0.854 | 0.040 |
| 218793_s_at | SCML1 | sex comb on midleg-like 1 (Drosophila) | 0.753 | 0.025 |
| 214180_at | MAN1C1 | mannosidase, alpha, class 1C, member 1 | 0.719 | 0.005 |
| 237347_at | NA | NA | 0.647 | 0.026 |
| | | | | |
| **Downregulated** | | | | |
| 228910_at | NA | NA | -0.589 | 0.040 |
| 226657_at | C17orf103 | chromosome 17 open reading frame 103 | -0.592 | 0.017 |
| 1564494_s_at | P4HB | prolyl 4-hydroxylase, beta polypeptide | -0.600 | 0.012 |
| 227089_at | COG5 | component of oligomeric golgi complex 5 | -0.631 | 0.026 |
| 208853_s_at | CANX | calnexin | -0.680 | 0.012 |
| 227114_at | RNF214 | ring finger protein 214 | -0.691 | 0.034 |
| 205321_at | EIF2S3 | eukaryotic translation initiation factor 2, subunit 3 | -0.704 | 0.024 |
| 205446_s_at | ATF2 | activating transcription factor 2 | -0.706 | 0.012 |
| 203629_s_at | COG5 | component of oligomeric golgi complex 5 | -0.725 | 0.024 |
| 203692_s_at | E2F3 | E2F transcription factor 3 | -0.830 | 0.030 |
| 229354_at | AHRR | aryl-hydrocarbon receptor repressor | -0.909 | 0.044 |
| 224480_s_at | AGPAT9 | 1-acylglycerol-3-phosphate O-acyltransferase 9 | -0.909 | 0.005 |
| 212665_at | TIPARP | TCDD-inducible poly(ADP-ribose) polymerase | -1.227 | 0.014 |
| 203470_s_at | PLEK | pleckstrin | -1.473 | 0.040 |

## Appendix D: Genes dysregulated in $CD8^+$ T cells from CLL patients

### Genes that are ≥ 3 fold up-regulated in $CD8^+$ T cells from CLL patients compared with healthy $CD8^+$ T cells

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 230137_at | TMEM155 | transmembrane protein 155 | 3.195 | 0.002 |
| 232629_at | PROK2 | prokineticin 2 | 2.945 | 0.041 |
| 210354_at | IFNG | interferon, gamma | 2.527 | 0.004 |
| 220030_at | STYK1 | serine/threonine/tyrosine kinase 1 | 2.516 | 0.002 |
| 207229_at | KLRA1 | killer cell lectin-like receptor subfamily A, member 1 | 2.404 | 0.000 |
| 210321_at | GZMH | granzyme H (cathepsin G-like 2, protein h-CCPX) | 2.366 | 0.012 |
| 229937_x_at | LILRB1 | leukocyte immunoglobulin-like receptor, subfamily B, 1 | 2.280 | 0.008 |
| 227478_at | SETBP1 | SET binding protein 1 | 2.240 | 0.017 |
| 227769_at | NA | NA | 2.213 | 0.038 |
| 221696_s_at | STYK1 | serine/threonine/tyrosine kinase 1 | 2.062 | 0.004 |
| 212810_s_at | SLC1A4 | solute carrier family , member 4 | 1.981 | 0.002 |
| 1555579_s_at | PTPRM | protein tyrosine phosphatase, receptor type, M | 1.924 | 0.001 |
| 241394_at | NA | NA | 1.900 | 0.006 |
| 238600_at | JAKMIP1 | janus kinase and microtubule interacting protein 1 | 1.863 | 0.003 |
| 227792_at | ITPRIPL2 | inositol-triphosphate receptor interacting protein-like 2 | 1.854 | 0.010 |
| 203329_at | PTPRM | protein tyrosine phosphatase, receptor type, M | 1.821 | 0.001 |
| 1555465_at | MCOLN2 | mucolipin 2 | 1.771 | 0.005 |
| 203603_s_at | ZEB2 | zinc finger E-box binding homeobox 2 | 1.688 | 0.014 |
| 219386_s_at | SLAMF8 | SLAM family member 8 | 1.683 | 0.019 |
| 227915_at | ASB2 | ankyrin repeat and SOCS box-containing 2 | 1.679 | 0.008 |
| 1558102_at | NA | NA | 1.672 | 0.009 |
| 209610_s_at | SLC1A4 | solute carrier family 1, member 4 | 1.648 | 0.009 |
| 205660_at | OASL | 2'-5'-oligoadenylate synthetase-like | 1.639 | 0.019 |
| 212811_x_at | SLC1A4 | solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 | 1.635 | 0.007 |
| 232504_at | 285628 | hypothetical protein LOC285628 | 1.626 | 0.011 |
| 228438_at | 100132891 | hypothetical protein LOC100132891 | 1.626 | 0.009 |
| 240054_at | NA | NA | 1.598 | 0.000 |
| 209032_s_at | CADM1 | cell adhesion molecule 1 | 1.590 | 0.014 |

## Genes that are ≥ 3 fold down-regulated in CD8$^+$ T cells from CLL patients compared with healthy CD8$^+$ T cells

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 202917_s_at | S100A8 | S100 calcium binding protein A8 | 8.506 | 0.000 |
| 205033_s_at | NA | NA | 7.442 | 0.002 |
| 203535_at | S100A9 | S100 calcium binding protein A9 | 7.223 | 0.000 |
| 202018_s_at | LTF | lactotransferrin | 6.906 | 0.000 |
| 209116_x_at | HBB | hemoglobin, beta | 6.764 | 0.002 |
| 211696_x_at | HBB | hemoglobin, beta | 6.510 | 0.003 |
| 1555745_a_at | LYZ | lysozyme (renal amyloidosis) | 6.281 | 0.000 |
| 217232_x_at | HBB | hemoglobin, beta | 6.256 | 0.002 |
| 209771_x_at | CD24 | CD24 molecule | 6.026 | 0.000 |
| 206676_at | CEACAM8 | carcinoembryonic antigen-related cell adhesion molecule 8 | 5.966 | 0.000 |
| 216379_x_at | CD24 | CD24 molecule | 5.796 | 0.000 |
| 205159_at | CSF2RB | colony stimulating factor 2 receptor, beta, low-affinity | 5.471 | 0.000 |
| 213975_s_at | LYZ | lysozyme (renal amyloidosis) | 5.396 | 0.000 |
| 212531_at | LCN2 | lipocalin 2 | 5.237 | 0.002 |
| 266_s_at | CD24 | CD24 molecule | 5.185 | 0.000 |
| 214414_x_at | NA | NA | 5.142 | 0.013 |
| 217414_x_at | NA | NA | 5.011 | 0.010 |
| 201669_s_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 5.007 | 0.000 |
| 209458_x_at | NA | NA | 4.982 | 0.010 |
| 210254_at | MS4A3 | membrane-spanning 4-domains, subfamily A, member 3 | 4.925 | 0.000 |
| 207269_at | DEFA4 | defensin, alpha 4, corticostatin | 4.909 | 0.002 |
| 210244_at | CAMP | cathelicidin antimicrobial peptide | 4.908 | 0.002 |
| 204018_x_at | NA | NA | 4.882 | 0.009 |
| 203923_s_at | CYBB | cytochrome b-245, beta polypeptide | 4.880 | 0.000 |
| 211745_x_at | NA | NA | 4.823 | 0.013 |
| 205863_at | S100A12 | S100 calcium binding protein A12 | 4.823 | 0.015 |
| 205119_s_at | FPR1 | formyl peptide receptor 1 | 4.715 | 0.000 |
| 202391_at | BASP1 | brain abundant, membrane attached signal protein 1 | 4.679 | 0.000 |
| 211699_x_at | NA | NA | 4.654 | 0.010 |
| 208650_s_at | CD24 | CD24 molecule | 4.631 | 0.000 |
| 206207_at | CLC | Charcot-Leyden crystal protein | 4.625 | 0.003 |
| 208651_x_at | CD24 | CD24 molecule | 4.602 | 0.000 |
| 207861_at | CCL22 | chemokine (C-C motif) ligand 22 | 4.521 | 0.000 |
| 211657_at | CEACAM6 | carcinoembryonic antigen-related cell adhesion molecule 6 | 4.467 | 0.003 |
| 207802_at | CRISP3 | cysteine-rich secretory protein 3 | 4.336 | 0.012 |
| 211734_s_at | FCER1A | Fc fragment of IgE, high affinity I, alpha polypeptide | 4.144 | 0.000 |
| 204971_at | CSTA | cystatin A (stefin A) | 4.140 | 0.000 |
| 205557_at | BPI | bactericidal/permeability-increasing protein | 4.138 | 0.002 |
| 217764_s_at | RAB31 | RAB31, member RAS oncogene family | 4.067 | 0.000 |
| 1552348_at | PRSS33 | protease, serine, 33 | 3.979 | 0.000 |
| 212592_at | IGJ | immunoglobulin J polypeptide | 3.887 | 0.017 |
| 205624_at | CPA3 | carboxypeptidase A3 (mast cell) | 3.874 | 0.003 |
| 212768_s_at | OLFM4 | olfactomedin 4 | 3.855 | 0.015 |

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 223809_at | RGS18 | regulator of G-protein signaling 18 | 3.831 | 0.000 |
| 208304_at | CCR3 | chemokine (C-C motif) receptor 3 | 3.761 | 0.000 |
| 231688_at | MMP8 | matrix metallopeptidase 8 (neutrophil collagenase) | 3.755 | 0.010 |
| 1554892_a_at | MS4A3 | membrane-spanning 4-domains, subfamily A, member 3 | 3.747 | 0.001 |
| 214677_x_at | NA | NA | 3.646 | 0.017 |
| 217763_s_at | RAB31 | RAB31, member RAS oncogene family | 3.633 | 0.000 |
| 209395_at | CHI3L1 | chitinase 3-like 1 (cartilage glycoprotein-39) | 3.616 | 0.008 |
| 209710_at | GATA2 | GATA binding protein 2 | 3.598 | 0.000 |
| 204959_at | MNDA | myeloid cell nuclear differentiation antigen | 3.514 | 0.005 |
| 217762_s_at | RAB31 | RAB31, member RAS oncogene family | 3.473 | 0.000 |
| 202589_at | TYMS | thymidylate synthetase | 3.425 | 0.001 |
| 204351_at | S100P | S100 calcium binding protein P | 3.406 | 0.002 |
| 209369_at | ANXA3 | annexin A3 | 3.371 | 0.011 |
| 1552386_at | GAPT | GRB2-binding adaptor protein, transmembrane | 3.289 | 0.000 |
| 227889_at | LPCAT2 | lysophosphatidylcholine acyltransferase 2 | 3.279 | 0.000 |
| 204232_at | FCER1G | Fc fragment of IgE, high affinity I, gamma polypeptide | 3.277 | 0.001 |
| 209949_at | NCF2 | neutrophil cytosolic factor 2 | 3.253 | 0.000 |
| 210029_at | IDO1 | indoleamine 2,3-dioxygenase 1 | 3.251 | 0.000 |
| 201163_s_at | IGFBP7 | insulin-like growth factor binding protein 7 | 3.250 | 0.002 |
| 209160_at | AKR1C3 | aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) | 3.249 | 0.003 |
| 1553177_at | SH2D1B | SH2 domain containing 1B | 3.229 | 0.007 |
| 217022_s_at | NA | NA | 3.179 | 0.024 |
| 1554899_s_at | FCER1G | Fc fragment of IgE, high affinity I, gamma polypeptide | 3.108 | 0.001 |
| 206177_s_at | ARG1 | arginase, liver | 3.070 | 0.025 |
| 202990_at | PYGL | phosphorylase, glycogen, liver | 3.045 | 0.000 |
| 204446_s_at | ALOX5 | arachidonate 5-lipoxygenase | 3.026 | 0.013 |
| 214370_at | S100A8 | S100 calcium binding protein A8 | 3.017 | 0.000 |
| 201670_s_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 2.839 | 0.002 |
| 208470_s_at | NA | NA | 2.794 | 0.034 |
| 204105_s_at | NRCAM | neuronal cell adhesion molecule | 2.718 | 0.025 |
| 226068_at | SYK | spleen tyrosine kinase | 2.684 | 0.001 |
| 206834_at | HBD | hemoglobin, delta | 2.672 | 0.000 |
| 206637_at | P2RY14 | purinergic receptor P2Y, G-protein coupled, 14 | 2.645 | 0.004 |
| 220001_at | PADI4 | peptidyl arginine deiminase, type IV | 2.623 | 0.006 |
| 202859_x_at | IL8 | interleukin 8 | 2.612 | 0.045 |
| 201425_at | ALDH2 | aldehyde dehydrogenase 2 family (mitochondrial) | 2.609 | 0.000 |
| 203757_s_at | CEACAM6 | carcinoembryonic antigen-related cell adhesion molecule 6 | 2.608 | 0.018 |
| 201890_at | RRM2 | ribonucleotide reductase M2 | 2.595 | 0.005 |
| 205513_at | TCN1 | transcobalamin I binding protein, R binder family | 2.571 | 0.008 |
| 202503_s_at | KIAA0101 | KIAA0101 | 2.541 | 0.005 |
| 206157_at | PTX3 | pentraxin-related gene, rapidly induced by IL-1 beta | 2.522 | 0.000 |
| 228170_at | OLIG1 | oligodendrocyte transcription factor 1 | 2.521 | 0.001 |
| 233401_at | NA | NA | 2.463 | 0.003 |
| 203973_s_at | CEBPD | CCAAT/enhancer binding protein (C/EBP), delta | 2.461 | 0.001 |
| 244313_at | CR1 | complement component (3b/4b) receptor 1 | 2.387 | 0.007 |
| 206697_s_at | HP | haptoglobin | 2.387 | 0.032 |

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 201417_at | SOX4 | SRY (sex determining region Y)-box 4 | 2.363 | 0.009 |
| 203021_at | SLPI | secretory leukocyte peptidase inhibitor | 2.347 | 0.017 |
| 201110_s_at | THBS1 | thrombospondin 1 | 2.334 | 0.001 |
| 227647_at | KCNE3 | potassium voltage-gated channel, Isk-related family, 3 | 2.303 | 0.011 |
| 225897_at | MARCKS | myristoylated alanine-rich protein kinase C substrate | 2.300 | 0.006 |
| 228854_at | NA | NA | 2.286 | 0.005 |
| 200696_s_at | GSN | gelsolin (amyloidosis, Finnish type) | 2.281 | 0.000 |
| 225710_at | GNB4 | guanine nucleotide binding protein, beta polypeptide 4 | 2.273 | 0.024 |
| 1552398_a_at | NA | NA | 2.256 | 0.037 |
| 207540_s_at | SYK | spleen tyrosine kinase | 2.249 | 0.001 |
| 206310_at | SPINK2 | serine peptidase inhibitor, Kazal type 2 | 2.228 | 0.004 |
| 229934_at | NA | NA | 2.228 | 0.013 |
| 203753_at | TCF4 | transcription factor 4 | 2.187 | 0.020 |
| 227762_at | NA | NA | 2.137 | 0.005 |
| 203921_at | CHST2 | carbohydrate (N-acetylglucosamine-6-O sulfotransferase 2 | 2.132 | 0.001 |
| 242509_at | NA | NA | 2.127 | 0.003 |
| 231579_s_at | TIMP2 | TIMP metallopeptidase inhibitor 2 | 2.121 | 0.029 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 2.120 | 0.037 |
| 201005_at | CD9 | CD9 molecule | 2.105 | 0.003 |
| 226817_at | DSC2 | desmocollin 2 | 2.097 | 0.007 |
| 208892_s_at | DUSP6 | dual specificity phosphatase 6 | 2.073 | 0.014 |
| 205051_s_at | KIT | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene | 2.070 | 0.017 |
| 206522_at | MGAM | maltase-glucoamylase (alpha-glucosidase) | 2.051 | 0.036 |
| 231771_at | GJB6 | gap junction protein, beta 6, 30kDa | 2.036 | 0.018 |
| 44790_s_at | C13orf18 | chromosome 13 open reading frame 18 | 2.016 | 0.034 |
| 223553_s_at | DOK3 | docking protein 3 | 2.011 | 0.013 |
| 212386_at | TCF4 | transcription factor 4 | 1.995 | 0.037 |
| 205016_at | TGFA | transforming growth factor, alpha | 1.984 | 0.006 |
| 202252_at | RAB13 | RAB13, member RAS oncogene family | 1.984 | 0.002 |
| 1553043_a_at | CD300LF | CD300 molecule-like family member f | 1.938 | 0.006 |
| 201462_at | SCRN1 | secernin 1 | 1.932 | 0.014 |
| 201739_at | SGK1 | serum/glucocorticoid regulated kinase 1 | 1.923 | 0.004 |
| 202897_at | SIRPA | signal-regulatory protein alpha | 1.922 | 0.010 |
| 217388_s_at | KYNU | kynureninase (L-kynurenine hydrolase) | 1.910 | 0.009 |
| 244741_s_at | NA | NA | 1.905 | 0.032 |
| 212192_at | KCTD12 | potassium channel tetramerisation domain containing 12 | 1.898 | 0.011 |
| 209921_at | SLC7A11 | solute carrier family 7, member 11 | 1.888 | 0.006 |
| 230793_at | LRRC16A | leucine rich repeat containing 16A | 1.880 | 0.019 |
| 208891_at | DUSP6 | dual specificity phosphatase 6 | 1.878 | 0.019 |
| 242794_at | MAML3 | mastermind-like 3 (Drosophila) | 1.865 | 0.002 |
| 210845_s_at | PLAUR | plasminogen activator, urokinase receptor | 1.853 | 0.008 |
| 222833_at | LPCAT2 | lysophosphatidylcholine acyltransferase 2 | 1.811 | 0.006 |
| 1560676_at | SIAH3 | seven in absentia homolog 3 (Drosophila) | 1.810 | 0.005 |
| 205147_x_at | NCF4 | neutrophil cytosolic factor 4, 40kDa | 1.807 | 0.014 |
| 204961_s_at | NA | NA | 1.804 | 0.006 |
| 210517_s_at | AKAP12 | A kinase (PRKA) anchor protein 12 | 1.785 | 0.015 |

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 204798_at | MYB | v-myb myeloblastosis viral oncogene homolog (avian) | 1.775 | 0.002 |
| 239272_at | MMP28 | matrix metallopeptidase 28 | 1.771 | 0.032 |
| 212188_at | KCTD12 | potassium channel tetramerisation domain containing 12 | 1.769 | 0.004 |
| 207857_at | LILRA2 | leukocyte immunoglobulin-like receptor, subfamily A, 2 | 1.766 | 0.046 |
| 209773_s_at | RRM2 | ribonucleotide reductase M2 | 1.757 | 0.045 |
| 1552349_a_at | PRSS33 | protease, serine, 33 | 1.744 | 0.010 |
| 205382_s_at | CFD | complement factor D (adipsin) | 1.733 | 0.016 |
| 207677_s_at | NCF4 | neutrophil cytosolic factor 4, 40kDa | 1.724 | 0.027 |
| 221581_s_at | LAT2 | linker for activation of T cells family, member 2 | 1.718 | 0.002 |
| 209772_s_at | CD24 | CD24 molecule | 1.708 | 0.037 |
| 204863_s_at | IL6ST | interleukin 6 signal transducer | 1.695 | 0.002 |
| 223623_at | C2orf40 | chromosome 2 open reading frame 40 | 1.680 | 0.048 |
| 220088_at | C5AR1 | complement component 5a receptor 1 | 1.679 | 0.027 |
| 228293_at | DEPDC7 | DEP domain containing 7 | 1.678 | 0.024 |
| 223028_s_at | SNX9 | sorting nexin 9 | 1.674 | 0.004 |
| 1552908_at | C1orf150 | chromosome 1 open reading frame 150 | 1.671 | 0.033 |
| 203139_at | DAPK1 | death-associated protein kinase 1 | 1.664 | 0.020 |
| 1552691_at | ARL11 | ADP-ribosylation factor-like 11 | 1.663 | 0.014 |
| 208637_x_at | ACTN1 | actinin, alpha 1 | 1.663 | 0.018 |
| 204057_at | IRF8 | interferon regulatory factor 8 | 1.653 | 0.002 |
| 204440_at | CD83 | CD83 molecule | 1.645 | 0.001 |
| 1562245_a_at | ZNF578 | zinc finger protein 578 | 1.638 | 0.014 |
| 244125_at | NA | NA | 1.637 | 0.035 |
| 231798_at | NOG | noggin | 1.623 | 0.043 |
| 231351_at | NA | NA | 1.622 | 0.002 |
| 228582_x_at | MALAT1 | metastasis associated lung adenocarcinoma transcript 1 | 1.617 | 0.033 |
| 201887_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 1.617 | 0.043 |
| 1558569_at | UNQ6228 | hypothetical LOC100131541 | 1.617 | 0.003 |
| 223423_at | GPR160 | G protein-coupled receptor 160 | 1.615 | 0.014 |
| 244414_at | NA | NA | 1.613 | 0.005 |
| 242405_at | NA | NA | 1.612 | 0.006 |
| 209191_at | TUBB6 | tubulin, beta 6 | 1.612 | 0.047 |
| 226884_at | LRRN1 | leucine rich repeat neuronal 1 | 1.611 | 0.004 |
| 219671_at | HPCAL4 | hippocalcin like 4 | 1.609 | 0.015 |
| 236858_s_at | RUNX2 | runt-related transcription factor 2 | 1.607 | 0.024 |
| 211000_s_at | IL6ST | interleukin 6 signal transducer | 1.606 | 0.004 |
| 227184_at | NA | NA | 1.597 | 0.006 |

## Appendix E: Genes dysregulated in $CD3^-CD56^+$ NK cells from CLL patients

### Genes that are ≥ 3 fold up-regulated in $CD3^-CD56^+$ NK cells from CLL patients compared with healthy $CD3^-CD56^+$ NK cells

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 238018_at | FAM150B | family with sequence similarity 150, member B | 3.229 | 0.022 |
| 220004_at | DDX43 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 43 | 2.886 | 0.010 |
| 219871_at | FLJ13197 | uncharacterized FLJ13197 | 2.490 | 0.008 |
| 220591_s_at | EFHC2 | EF-hand domain (C-terminal) containing 2 | 2.125 | 0.031 |
| 229952_at | NA | NA | 1.889 | 0.001 |
| 219412_at | RAB38 | RAB38, member RAS oncogene family | 1.742 | 0.007 |
| 204916_at | RAMP1 | receptor (G protein-coupled) activity modifying protein 1 | 1.732 | 0.007 |
| 229040_at | ITGB2-AS1 | ITGB2 antisense RNA 1 | 1.723 | 0.006 |
| 241278_at | NA | NA | 1.696 | 0.043 |
| 229041_s_at | ITGB2-AS1 | ITGB2 antisense RNA 1 | 1.592 | 0.001 |

### Genes that are ≥ 3 fold down-regulated in $CD3^-CD56^+$ NK cells from CLL patients compared with healthy $CD3^-CD56^+$ NK cells

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 204439_at | IFI44L | interferon-induced protein 44-like | 6.529 | 0.000 |
| 203153_at | IFIT1 | interferon-induced protein with tetratricopeptide repeats 1 | 4.212 | 0.000 |
| 213797_at | RSAD2 | radical S-adenosyl methionine domain containing 2 | 4.018 | 0.000 |
| 242625_at | RSAD2 | radical S-adenosyl methionine domain containing 2 | 3.598 | 0.000 |
| 205330_at | MN1 | meningioma (disrupted in balanced translocation) 1 | 3.454 | 0.010 |
| 236595_at | NA | NA | 3.313 | 0.005 |
| 214059_at | IFI44 | interferon-induced protein 44 | 3.237 | 0.000 |
| 218400_at | OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa | 3.201 | 0.000 |
| 205552_s_at | OAS1 | 2'-5'-oligoadenylate synthetase 1, 40/46kDa | 3.108 | 0.001 |
| 202086_at | MX1 | myxovirus resistance 1, interferon-inducible protein p78 | 3.061 | 0.004 |
| 229450_at | IFIT3 | interferon-induced protein with tetratricopeptide repeats 3 | 2.996 | 0.000 |
| 205479_s_at | PLAU | plasminogen activator, urokinase | 2.981 | 0.019 |
| 204747_at | IFIT3 | interferon-induced protein with tetratricopeptide repeats 3 | 2.935 | 0.001 |
| 214453_s_at | IFI44 | interferon-induced protein 44 | 2.870 | 0.000 |
| 232081_at | ABCG1 | ATP-binding cassette, sub-family G (WHITE), member 1 | 2.760 | 0.001 |
| 226702_at | CMPK2 | cytidine monophosphate kinase 2, mitochondrial | 2.663 | 0.000 |
| 230250_at | PTPRB | protein tyrosine phosphatase, receptor type, B | 2.656 | 0.003 |
| 202869_at | OAS1 | 2'-5'-oligoadenylate synthetase 1, 40/46kDa | 2.645 | 0.001 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 2.610 | 0.004 |
| 204415_at | IFI6 | interferon, alpha-inducible protein 6 | 2.540 | 0.001 |
| 222449_at | PMEPA1 | prostate transmembrane protein, androgen induced 1 | 2.505 | 0.003 |
| 212680_x_at | PPP1R14B | protein phosphatase 1, regulatory (inhibitor) subunit 14B | 2.443 | 0.002 |
| 219753_at | STAG3 | stromal antigen 3 | 2.413 | 0.005 |
| 214247_s_at | DKK3 | dickkopf 3 homolog (Xenopus laevis) | 2.400 | 0.027 |
| 242898_at | EIF2AK2 | eukaryotic translation initiation factor 2-alpha kinase 2 | 2.354 | 0.001 |
| 222154_s_at | SPATS2L | spermatogenesis associated, serine-rich 2-like | 2.235 | 0.005 |

| Probe ID | Symbol | Description | $Log_2$ FC | Adjusted P value |
|---|---|---|---|---|
| 230913_at | NA | NA | 2.231 | 0.036 |
| 217875_s_at | PMEPA1 | prostate transmembrane protein, androgen induced 1 | 2.202 | 0.012 |
| 219866_at | CLIC5 | chloride intracellular channel 5 | 2.198 | 0.009 |
| 205977_s_at | EPHA1 | EPH receptor A1 | 2.182 | 0.036 |
| 222450_at | PMEPA1 | prostate transmembrane protein, androgen induced 1 | 2.170 | 0.024 |
| 201465_s_at | JUN | jun proto-oncogene | 2.119 | 0.000 |
| 210001_s_at | SOCS1 | suppressor of cytokine signaling 1 | 2.086 | 0.001 |
| 217497_at | TYMP | thymidine phosphorylase | 2.054 | 0.001 |
| 219211_at | USP18 | ubiquitin specific peptidase 18 | 2.048 | 0.002 |
| 204567_s_at | ABCG1 | ATP-binding cassette, sub-family G (WHITE), member 1 | 2.022 | 0.012 |
| 235276_at | EPSTI1 | epithelial stromal interaction 1 (breast) | 1.978 | 0.000 |
| 201466_s_at | JUN | jun proto-oncogene | 1.978 | 0.000 |
| 219949_at | LRRC2 | leucine rich repeat containing 2 | 1.938 | 0.034 |
| 236439_at | NA | NA | 1.899 | 0.033 |
| 235301_at | KIAA1324L | KIAA1324-like | 1.892 | 0.021 |
| 209574_s_at | C18orf1 | chromosome 18 open reading frame 1 | 1.887 | 0.007 |
| 211434_s_at | CCRL2 | chemokine (C-C motif) receptor-like 2 | 1.880 | 0.030 |
| 204994_at | MX2 | myxovirus (influenza virus) resistance 2 (mouse) | 1.873 | 0.012 |
| 205660_at | OASL | 2'-5'-oligoadenylate synthetase-like | 1.870 | 0.000 |
| 204285_s_at | PMAIP1 | phorbol-12-myristate-13-acetate-induced protein 1 | 1.858 | 0.018 |
| 202468_s_at | CTNNAL1 | catenin (cadherin-associated protein), alpha-like 1 | 1.827 | 0.018 |
| 227609_at | EPSTI1 | epithelial stromal interaction 1 (breast) | 1.817 | 0.000 |
| 235916_at | YPEL4 | yippee-like 4 (Drosophila) | 1.810 | 0.016 |
| 210797_s_at | OASL | 2'-5'-oligoadenylate synthetase-like | 1.796 | 0.000 |
| 209573_s_at | C18orf1 | chromosome 18 open reading frame 1 | 1.777 | 0.014 |
| 204286_s_at | PMAIP1 | phorbol-12-myristate-13-acetate-induced protein 1 | 1.727 | 0.029 |
| 205483_s_at | ISG15 | ISG15 ubiquitin-like modifier | 1.724 | 0.019 |
| 219352_at | HERC6 | HECT and RLD domain containing E3 ubiquitin protein ligase family member 6 | 1.718 | 0.001 |
| 224802_at | NDFIP2 | Nedd4 family interacting protein 2 | 1.602 | 0.006 |
| 201464_x_at | JUN | jun proto-oncogene | 1.593 | 0.000 |

## Appendix F: Impact of lenalidomide on gene expression of CLL cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 204223_at | PRELP | proline/arginine-rich end leucine-rich repeat protein | 4.271 | 3.83E-09 |
| 1557049_at | BTBD19 | BTB (POZ) domain containing 19 | 3.589 | 4.66E-09 |
| 205987_at | CD1C | CD1c molecule | 3.467 | 1.65E-05 |
| 1559067_a_at | NA | NA | 3.249 | 4.23E-09 |
| 214228_x_at | TNFRSF4 | tumor necrosis factor receptor superfamily, 4 | 3.078 | 1.35E-09 |
| 230966_at | IL4I1 | interleukin 4 induced 1 | 3.058 | 1.83E-06 |
| 226264_at | SUSD1 | sushi domain containing 1 | 3.018 | 6.91E-08 |
| 236769_at | LOC158402 | uncharacterized LOC158402 | 3.013 | 9.33E-09 |
| 238365_s_at | C1orf228 | chromosome 1 open reading frame 228 | 2.961 | 3.79E-11 |
| 204103_at | CCL4 | chemokine (C-C motif) ligand 4 | 2.848 | 2.57E-05 |
| 202638_s_at | ICAM1 | intercellular adhesion molecule 1 | 2.785 | 4.62E-08 |
| 242520_s_at | C1orf228 | chromosome 1 open reading frame 228 | 2.777 | 1.24E-09 |
| 209785_s_at | PLA2G4C | phospholipase A2, group | 2.739 | 1.98E-07 |
| 223249_at | CLDN12 | claudin 12 | 2.733 | 2.66E-09 |
| 212458_at | SPRED2 | sprouty-related, EVH1 domain containing 2 | 2.677 | 5.19E-06 |
| 209757_s_at | MYCN | v-myc myelocytomatosis viral related oncogene | 2.626 | 2.53E-06 |
| 210056_at | RND1 | Rho family GTPase 1 | 2.597 | 1.11E-10 |
| 230787_at | NA | NA | 2.524 | 1.48E-11 |
| 229450_at | IFIT3 | interferon-induced with tetratricopeptide repeats 3 | 2.445 | 3.51E-03 |
| 213925_at | C1orf95 | chromosome 1 open reading frame 95 | 2.438 | 2.40E-05 |
| 1559066_at | NA | NA | 2.421 | 1.49E-07 |
| 228224_at | PRELP | proline/arginine-rich end leucine-rich repeat protein | 2.392 | 1.41E-04 |
| 205114_s_at | NA | NA | 2.356 | 1.02E-05 |
| 1556281_at | NA | NA | 2.312 | 1.90E-06 |
| 229127_at | JAM2 | junctional adhesion molecule 2 | 2.301 | 5.29E-07 |
| 235175_at | GBP4 | guanylate binding protein 4 | 2.280 | 2.40E-05 |
| 213348_at | CDKN1C | cyclin-dependent kinase inhibitor 1C (p57, Kip2) | 2.268 | 3.27E-07 |
| 207113_s_at | TNF | tumor necrosis factor | 2.256 | 8.97E-09 |
| 202637_s_at | ICAM1 | intercellular adhesion molecule 1 | 2.256 | 6.02E-09 |
| 207426_s_at | TNFSF4 | tumor necrosis factor (ligand) superfamily, member 4 | 2.255 | 2.27E-07 |
| 200983_x_at | CD59 | CD59 molecule, complement regulatory protein | 2.253 | 2.59E-16 |
| 209959_at | NR4A3 | nuclear receptor subfamily 4, group A, member 3 | 2.222 | 1.25E-04 |
| 204083_s_at | TPM2 | tropomyosin 2 (beta) | 2.199 | 4.72E-05 |
| 208370_s_at | RCAN1 | regulator of calcineurin 1 | 2.192 | 2.40E-08 |
| 230939_at | LOC100996457 | uncharacterized LOC100996457 | 2.185 | 1.11E-10 |
| 204026_s_at | ZWINT | ZW10 interactor | 2.128 | 1.15E-09 |
| 218400_at | OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa | 2.114 | 3.88E-06 |
| 201939_at | PLK2 | polo-like kinase 2 | 2.096 | 4.08E-07 |
| 209267_s_at | SLC39A8 | solute carrier family 39 (zinc transporter), member 8 | 2.081 | 7.54E-09 |
| 234284_at | GNG8 | guanine nucleotide binding protein, gamma 8 | 2.071 | 6.07E-05 |
| 215253_s_at | RCAN1 | regulator of calcineurin 1 | 2.054 | 5.06E-06 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 2.049 | 1.41E-04 |
| 200984_s_at | CD59 | CD59 molecule, complement regulatory protein | 2.032 | 5.78E-16 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 200985_s_at | CD59 | CD59 molecule, complement regulatory protein | 2.019 | 2.39E-16 |
| 239691_at | C12orf77 | chromosome 12 open reading frame 77 | 2.003 | 3.90E-05 |
| 209684_at | RIN2 | Ras and Rab interactor 2 | 1.967 | 4.74E-03 |
| 219213_at | JAM2 | junctional adhesion molecule 2 | 1.967 | 6.88E-05 |
| 206674_at | FLT3 | fms-related tyrosine kinase 3 | 1.959 | 3.00E-06 |
| 201059_at | CTTN | cortactin | 1.956 | 6.85E-06 |
| 202284_s_at | CDKN1A | cyclin-dependent kinase inhibitor 1A (p21, Cip1) | 1.950 | 9.63E-11 |
| 223159_s_at | NEK6 | NIMA (never in mitosis gene a)-related kinase 6 | 1.947 | 2.68E-05 |
| 212463_at | CD59 | CD59 molecule, complement regulatory protein | 1.913 | 1.28E-18 |
| 200951_s_at | CCND2 | cyclin D2 | 1.910 | 2.28E-05 |
| 206486_at | LAG3 | lymphocyte-activation gene 3 | 1.907 | 2.48E-04 |
| 206115_at | EGR3 | early growth response 3 | 1.887 | 1.78E-03 |
| 204422_s_at | FGF2 | fibroblast growth factor 2 (basic) | 1.878 | 1.03E-03 |
| 219869_s_at | SLC39A8 | solute carrier family 39 (zinc transporter), member 8 | 1.876 | 2.01E-08 |
| 205249_at | EGR2 | early growth response 2 | 1.848 | 1.11E-04 |
| 207691_x_at | ENTPD1 | ectonucleoside triphosphate diphosphohydrolase 1 | 1.845 | 1.75E-06 |
| 201693_s_at | EGR1 | early growth response 1 | 1.842 | 3.90E-05 |
| 226926_at | DMKN | dermokine | 1.841 | 2.49E-06 |
| 204622_x_at | NR4A2 | nuclear receptor subfamily 4, group A, member 2 | 1.839 | 2.81E-04 |
| 219255_x_at | IL17RB | interleukin 17 receptor B | 1.830 | 4.65E-04 |
| 219753_at | STAG3 | stromal antigen 3 | 1.830 | 3.51E-08 |
| 209129_at | TRIP6 | thyroid hormone receptor interactor 6 | 1.815 | 1.41E-11 |
| 223967_at | ANGPTL6 | angiopoietin-like 6 | 1.809 | 8.11E-08 |
| 202446_s_at | PLSCR1 | phospholipid scramblase 1 | 1.797 | 2.81E-07 |
| 204493_at | BID | BH3 interacting domain death agonist | 1.793 | 3.84E-05 |
| 216248_s_at | NR4A2 | nuclear receptor subfamily 4, group A, member 2 | 1.775 | 2.57E-04 |
| 210904_s_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 1.759 | 1.50E-04 |
| 209901_x_at | AIF1 | allograft inflammatory factor 1 | 1.727 | 1.71E-03 |
| 209474_s_at | ENTPD1 | ectonucleoside triphosphate diphosphohydrolase 1 | 1.721 | 5.97E-06 |
| 226517_at | BCAT1 | branched chain amino-acid transaminase 1, cytosolic | 1.713 | 1.74E-02 |
| 223845_at | VSIG10 | V-set and immunoglobulin domain containing 10 | 1.711 | 7.63E-04 |
| 228585_at | ENTPD1 | ectonucleoside triphosphate diphosphohydrolase 1 | 1.696 | 1.30E-06 |
| 211725_s_at | NA | NA | 1.695 | 3.05E-05 |
| 201887_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 1.694 | 1.34E-05 |
| 222746_s_at | BSPRY | B-box and SPRY domain containing | 1.691 | 2.39E-06 |
| 227404_s_at | EGR1 | early growth response 1 | 1.688 | 6.35E-04 |
| 220770_s_at | C5orf54 | chromosome 5 open reading frame 54 | 1.688 | 1.07E-09 |
| 202252_at | RAB13 | RAB13, member RAS oncogene family | 1.679 | 6.61E-08 |
| 227143_s_at | BID | BH3 interacting domain death agonist | 1.675 | 5.66E-06 |
| 1565817_at | IKZF1 | IKAROS family zinc finger 1 (Ikaros) | 1.666 | 1.78E-06 |
| 1554712_a_at | GLYATL2 | glycine-N-acyltransferase-like 2 | 1.666 | 4.65E-04 |
| 205204_at | NMB | neuromedin B | 1.662 | 1.30E-04 |
| 210481_s_at | CLEC4M | C-type lectin domain family 4, member M | 1.653 | 3.14E-04 |
| 204621_s_at | NR4A2 | nuclear receptor subfamily 4, group A, member 2 | 1.652 | 1.78E-04 |
| 237837_at | NA | NA | 1.641 | 6.19E-06 |
| 216973_s_at | HOXB7 | homeobox B7 | 1.622 | 1.04E-05 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 225285_at | BCAT1 | branched chain amino-acid transaminase 1, cytosolic | 1.615 | 2.89E-02 |
| 202430_s_at | PLSCR1 | phospholipid scramblase 1 | 1.611 | 1.11E-06 |
| 211612_s_at | IL13RA1 | interleukin 13 receptor, alpha 1 | 1.605 | 2.01E-04 |
| 224374_s_at | EMILIN2 | elastin microfibril interfacer 2 | 1.597 | 2.31E-09 |
| 207399_at | BFSP2 | beaded filament structural protein 2, phakinin | 1.596 | 7.05E-05 |
| 1553991_s_at | VSIG10 | V-set and immunoglobulin domain containing 10 | 1.594 | 6.92E-04 |
| 204068_at | STK3 | serine/threonine kinase 3 | 1.585 | 1.94E-07 |
| 218792_s_at | BSPRY | B-box and SPRY domain containing | 1.584 | 2.09E-07 |
| 220066_at | NOD2 | nucleotide-binding oligomerization domain containing 2 | 1.583 | 7.52E-07 |
| **Downregulated** | | | | |
| 205489_at | CRYM | crystallin, mu | 2.6092 | 1.21E-07 |
| 219518_s_at | NA | NA | 2.3277 | 7.67E-09 |
| 206312_at | GUCY2C | guanylate cyclase 2C | 2.0830 | 4.37E-05 |
| 216598_s_at | CCL2 | chemokine (C-C motif) ligand 2 | 2.0740 | 2.54E-03 |
| 206865_at | HRK | harakiri, BCL2 interacting protein | 2.0711 | 7.25E-06 |
| 229552_at | LOC283454 | uncharacterized LOC283454 | 2.0348 | 2.37E-05 |
| 213906_at | MYBL1 | v-myb myeloblastosis oncogene homolog-like 1 | 1.9810 | 6.34E-05 |
| 219517_at | NA | NA | 1.9666 | 2.47E-08 |
| 209598_at | PNMA2 | paraneoplastic Ma antigen 2 | 1.9469 | 8.69E-03 |
| 201005_at | CD9 | CD9 molecule | 1.9464 | 1.92E-04 |
| 202917_s_at | S100A8 | S100 calcium binding protein A8 | 1.8944 | 6.78E-03 |
| 237187_at | NA | NA | 1.8227 | 6.52E-06 |
| 206864_s_at | HRK | harakiri, BCL2 interacting protein | 1.7908 | 5.57E-06 |
| 209082_s_at | COL18A1 | collagen, type XVIII, alpha 1 | 1.7775 | 2.91E-03 |
| 201110_s_at | THBS1 | thrombospondin 1 | 1.7702 | 2.16E-02 |
| 219840_s_at | TCL6 | T-cell leukemia/lymphoma 6 (non-protein coding) | 1.7341 | 1.29E-08 |
| 1561363_a_at | NA | NA | 1.7336 | 4.51E-07 |
| 243469_at | NA | NA | 1.7296 | 9.78E-09 |
| 233142_at | NA | NA | 1.7043 | 1.57E-03 |
| 1564154_at | NA | NA | 1.6893 | 2.77E-05 |
| 235694_at | TCFL5 | transcription factor-like 5 (basic helix-loop-helix) | 1.6778 | 6.49E-09 |
| 210036_s_at | KCNH2 | potassium voltage-gated channel, subfamily H, 2 | 1.6613 | 2.54E-05 |
| 204681_s_at | RAPGEF5 | Rap guanine nucleotide exchange factor (GEF) 5 | 1.6487 | 2.75E-02 |
| 207087_x_at | ANK1 | ankyrin 1, erythrocytic | 1.6439 | 9.16E-06 |
| 226733_at | PFKFB2 | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 | 1.6302 | 5.36E-06 |
| 233951_at | NA | NA | 1.6237 | 8.74E-07 |
| 243309_at | FLJ27352 | uncharacterized LOC145788 | 1.6116 | 2.82E-09 |
| 213839_at | CLMN | calmin (calponin-like, transmembrane) | 1.5965 | 5.29E-06 |

## Appendix G: Impact of lenalidomide on gene expression of healthy B cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 215784_at | CD1E | CD1e molecule | 5.516 | 2.67E-18 |
| 209324_s_at | RGS16 | regulator of G-protein signaling 16 | 3.258 | 1.66E-05 |
| 200665_s_at | SPARC | secreted protein, acidic, cysteine-rich (osteonectin) | 3.073 | 2.55E-06 |
| 209325_s_at | RGS16 | regulator of G-protein signaling 16 | 2.708 | 2.00E-07 |
| 220066_at | NOD2 | nucleotide-binding oligomerization domain 2 | 2.381 | 2.03E-09 |
| 205968_at | KCNS3 | potassium voltage-gated channel, subfamily S, 3 | 2.299 | 2.10E-09 |
| 202450_s_at | CTSK | cathepsin K | 2.240 | 4.81E-04 |
| 210056_at | RND1 | Rho family GTPase 1 | 2.146 | 2.02E-08 |
| 242520_s_at | C1orf228 | chromosome 1 open reading frame 228 | 2.140 | 6.17E-07 |
| 234994_at | TMEM200A | transmembrane protein 200A | 2.098 | 1.04E-06 |
| 232397_at | NA | NA | 2.092 | 1.47E-06 |
| 207426_s_at | TNFSF4 | tumor necrosis factor superfamily, member 4 | 2.040 | 4.71E-06 |
| 210029_at | IDO1 | indoleamine 2,3-dioxygenase 1 | 2.023 | 5.12E-05 |
| 223249_at | CLDN12 | claudin 12 | 1.993 | 3.01E-06 |
| 232267_at | GPR133 | G protein-coupled receptor 133 | 1.972 | 6.60E-09 |
| 234284_at | GNG8 | guanine nucleotide binding protein, gamma 8 | 1.905 | 6.11E-04 |
| 238365_s_at | C1orf228 | chromosome 1 open reading frame 228 | 1.829 | 1.60E-06 |
| 208592_s_at | CD1E | CD1e molecule | 1.816 | 2.03E-09 |
| 202085_at | TJP2 | tight junction protein 2 | 1.782 | 9.78E-05 |
| 204932_at | TNFRSF11B | tumor necrosis factor receptor superfamily, 11b | 1.774 | 4.31E-04 |
| 203915_at | CXCL9 | chemokine (C-X-C motif) ligand 9 | 1.756 | 2.32E-04 |
| 224402_s_at | FCRL4 | Fc receptor-like 4 | 1.700 | 6.68E-03 |
| 204430_s_at | SLC2A5 | solute carrier family 2, member 5 | 1.681 | 2.94E-04 |
| 235885_at | P2RY12 | purinergic receptor P2Y, G-protein coupled, 12 | 1.667 | 2.22E-03 |
| 202481_at | DHRS3 | dehydrogenase/reductase (SDR family) member 3 | 1.664 | 1.02E-04 |
| 218094_s_at | NA | NA | 1.635 | 8.76E-07 |
| 212364_at | MYO1B | myosin IB | 1.624 | 9.44E-06 |
| 210643_at | TNFSF11 | tumor necrosis factor superfamily, member 11 | 1.603 | 2.41E-06 |
| 212655_at | ZCCHC14 | zinc finger, CCHC domain containing 14 | 1.596 | 8.89E-04 |
| 206618_at | IL18R1 | interleukin 18 receptor 1 | 1.583 | 3.95E-06 |
| **Downregulated** | | | | |
| 216598_s_at | CCL2 | chemokine (C-C motif) ligand 2 | 2.293 | 3.46E-03 |
| 204614_at | SERPINB2 | serpin peptidase inhibitor, clade B, member 2 | 2.098 | 2.23E-02 |
| 201108_s_at | THBS1 | thrombospondin 1 | 1.894 | 1.42E-02 |
| 240633_at | DOK7 | docking protein 7 | 1.866 | 8.95E-06 |
| 211429_s_at | SERPINA1 | serpin peptidase inhibitor, clade A, member 1 | 1.815 | 2.54E-03 |
| 209189_at | FOS | FBJ murine osteosarcoma viral oncogene homolog | 1.800 | 2.49E-03 |
| 229656_s_at | EML6 | echinoderm microtubule associated protein like 6 | 1.760 | 2.32E-04 |
| 206244_at | CR1 | complement component (3b/4b) receptor 1 | 1.752 | 2.75E-09 |
| 1555756_a_at | CLEC7A | C-type lectin domain family 7, member A | 1.733 | 1.59E-02 |
| 205237_at | FCN1 | ficolin (collagen/fibrinogen domain containing) 1 | 1.730 | 1.84E-02 |
| 230550_at | MS4A6A | membrane-spanning 4-domains, subfamily A, 6A | 1.697 | 9.62E-03 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 201109_s_at | THBS1 | thrombospondin 1 | 1.696 | 1.99E-02 |
| 201131_s_at | CDH1 | cadherin 1, type 1, E-cadherin (epithelial) | 1.674 | 7.43E-04 |
| 38487_at | STAB1 | stabilin 1 | 1.630 | 9.52E-03 |
| 201325_s_at | EMP1 | epithelial membrane protein 1 | 1.622 | 3.86E-03 |
| 202833_s_at | SERPINA1 | serpin peptidase inhibitor, clade A, member 1 | 1.595 | 1.98E-02 |
| 209875_s_at | SPP1 | secreted phosphoprotein 1 | 1.581 | 4.73E-02 |

## Appendix H: Impact of lenalidomide on gene expression of CLL CD4$^+$ T cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 204165_at | WASF1 | WAS protein family, member 1 | 4.443 | 1.42E-16 |
| 223249_at | CLDN12 | claudin 12 | 3.445 | 2.16E-18 |
| 244397_at | NA | NA | 3.192 | 6.89E-17 |
| 1565817_at | IKZF1 | IKAROS family zinc finger 1 (Ikaros) | 3.016 | 1.03E-16 |
| 240581_at | LOC644135 | uncharacterized LOC644135 | 2.849 | 2.85E-13 |
| 230939_at | LOC100996457 | uncharacterized LOC100996457 | 2.804 | 5.96E-09 |
| 232397_at | NA | NA | 2.761 | 4.40E-14 |
| 240103_at | LOC100996457 | uncharacterized LOC100996457 | 2.666 | 8.18E-08 |
| 213238_at | ATP10D | ATPase, class V, type 10D | 2.620 | 4.82E-13 |
| 213566_at | RNASE6 | ribonuclease, RNase A family, k6 | 2.612 | 3.39E-09 |
| 203476_at | TPBG | trophoblast glycoprotein | 2.606 | 2.20E-08 |
| 236787_at | NA | NA | 2.528 | 4.78E-09 |
| 202893_at | UNC13B | unc-13 homolog B (C. elegans) | 2.389 | 8.30E-12 |
| 1565579_at | NA | NA | 2.383 | 2.38E-08 |
| 219179_at | DACT1 | dapper, antagonist of beta-catenin, homolog 1 | 2.371 | 1.05E-08 |
| 225308_s_at | TANC1 | tetratricopeptide, ankyrin repeat and coiled-coil 1 | 2.356 | 9.89E-08 |
| 235412_at | ARHGEF7 | Rho guanine nucleotide exchange factor (GEF) 7 | 2.278 | 1.44E-09 |
| 207113_s_at | TNF | tumor necrosis factor | 2.174 | 1.74E-06 |
| 220770_s_at | C5orf54 | chromosome 5 open reading frame 54 | 2.154 | 1.60E-09 |
| 201743_at | CD14 | CD14 molecule | 2.001 | 2.04E-06 |
| 229450_at | IFIT3 | interferon-induced protein tetratricopeptide repeats 3 | 1.920 | 1.76E-02 |
| 225081_s_at | CDCA7L | cell division cycle associated 7-like | 1.870 | 6.47E-12 |
| 224374_s_at | EMILIN2 | elastin microfibril interfacer 2 | 1.831 | 2.74E-12 |
| 228027_at | GPRASP2 | G protein receptor associated sorting protein 2 | 1.801 | 8.43E-11 |
| 238029_s_at | SLC16A14 | solute carrier family 16, member 14 | 1.774 | 3.38E-06 |
| 223095_at | MARVELD1 | MARVEL domain containing 1 | 1.766 | 7.39E-13 |
| 204083_s_at | TPM2 | tropomyosin 2 (beta) | 1.739 | 1.52E-05 |
| 206380_s_at | CFP | complement factor properdin | 1.730 | 8.36E-11 |
| 227522_at | CMBL | carboxymethylenebutenolidase homolog | 1.725 | 6.49E-09 |
| 217890_s_at | PARVA | parvin, alpha | 1.718 | 2.81E-07 |
| 217196_s_at | CAMSAP2 | calmodulin regulated spectrin-associated protein 2 | 1.707 | 5.98E-13 |
| 212765_at | CAMSAP2 | calmodulin regulated spectrin-associated protein 2 | 1.678 | 8.26E-12 |
| 220358_at | BATF3 | basic leucine zipper transcription factor, ATF-like 3 | 1.619 | 1.10E-05 |
| 230301_at | NA | NA | 1.613 | 3.36E-05 |
| 209129_at | TRIP6 | thyroid hormone receptor interactor 6 | 1.595 | 1.40E-12 |
| 206600_s_at | SLC16A5 | solute carrier family 16, member 5 | 1.582 | 1.96E-10 |
| **Downregulated** | | | | |
| 205733_at | BLM | Bloom syndrome, RecQ helicase-like | 2.251 | 3.17E-08 |
| 220131_at | FXYD7 | FXYD domain containing ion transport regulator 7 | 1.955 | 1.24E-06 |
| 210448_s_at | P2RX5 | purinergic receptor P2X, ligand-gated ion channel, 5 | 1.837 | 2.06E-11 |
| 222307_at | LOC282997 | uncharacterized LOC282997 | 1.772 | 7.74E-17 |
| 242509_at | NA | NA | 1.661 | 1.86E-07 |
| 336_at | TBXA2R | thromboxane A2 receptor | 1.600 | 4.05E-05 |

## Appendix I: Impact of lenalidomide on gene expression of CLL $CD8^+$ T cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 223249_at | CLDN12 | claudin 12 | 3.328 | 4.79E-16 |
| 232397_at | NA | NA | 3.265 | 1.60E-08 |
| 240581_at | LOC644135 | uncharacterized LOC644135 | 3.230 | 2.04E-12 |
| 225710_at | GNB4 | guanine nucleotide binding prot. beta polypeptide 4 | 3.001 | 3.92E-04 |
| 204165_at | WASF1 | WAS protein family, member 1 | 2.907 | 1.27E-07 |
| 229450_at | IFIT3 | interferon-induced protein tetratricopeptide repeats 3 | 2.619 | 4.14E-02 |
| 219386_s_at | SLAMF8 | SLAM family member 8 | 2.600 | 8.72E-07 |
| 236787_at | NA | NA | 2.593 | 6.91E-06 |
| 244397_at | NA | NA | 2.529 | 2.04E-12 |
| 213566_at | RNASE6 | ribonuclease, RNase A family, k6 | 2.427 | 1.63E-04 |
| 219385_at | SLAMF8 | SLAM family member 8 | 2.345 | 1.93E-06 |
| 204083_s_at | TPM2 | tropomyosin 2 (beta) | 2.213 | 9.88E-08 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 2.192 | 4.51E-03 |
| 1565817_at | IKZF1 | IKAROS family zinc finger 1 (Ikaros) | 2.159 | 5.38E-08 |
| 235574_at | GBP4 | guanylate binding protein 4 | 2.131 | 1.03E-04 |
| 213113_s_at | SLC43A3 | solute carrier family 43, member 3 | 2.128 | 2.31E-11 |
| 218400_at | OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa | 2.087 | 1.35E-02 |
| 200678_x_at | GRN | granulin | 2.025 | 1.61E-06 |
| 208923_at | CYFIP1 | cytoplasmic FMR1 interacting protein 1 | 2.005 | 4.35E-07 |
| 220358_at | BATF3 | basic leucine zipper transcription factor, ATF-like 3 | 1.979 | 5.08E-03 |
| 224374_s_at | EMILIN2 | elastin microfibril interfacer 2 | 1.965 | 1.50E-09 |
| 216041_x_at | GRN | granulin | 1.955 | 1.45E-06 |
| 207111_at | EMR1 | egf-like module containing, mucin- receptor-like 1 | 1.934 | 7.53E-04 |
| 235175_at | GBP4 | guanylate binding protein 4 | 1.878 | 8.48E-05 |
| 211284_s_at | GRN | granulin | 1.873 | 8.54E-07 |
| 219403_s_at | HPSE | heparanase | 1.828 | 2.44E-05 |
| 1552691_at | ARL11 | ADP-ribosylation factor-like 11 | 1.815 | 4.40E-04 |
| 204068_at | STK3 | serine/threonine kinase 3 | 1.772 | 5.08E-07 |
| 225081_s_at | CDCA7L | cell division cycle associated 7-like | 1.759 | 1.28E-05 |
| 207113_s_at | TNF | tumor necrosis factor | 1.748 | 2.04E-06 |
| 228937_at | LACC1 | laccase domain containing 1 | 1.739 | 1.14E-03 |
| 213238_at | ATP10D | ATPase, class V, type 10D | 1.736 | 1.93E-05 |
| 227438_at | ALPK1 | alpha-kinase 1 | 1.718 | 9.66E-04 |
| 1565579_at | NA | NA | 1.711 | 7.09E-08 |
| 225285_at | BCAT1 | branched chain amino-acid transaminase 1, cytosolic | 1.692 | 7.37E-04 |
| 201952_at | ALCAM | activated leukocyte cell adhesion molecule | 1.687 | 1.29E-08 |
| 206637_at | P2RY14 | purinergic receptor P2Y, G-protein coupled, 14 | 1.678 | 1.18E-03 |
| 230511_at | CREM | cAMP responsive element modulator | 1.631 | 4.32E-04 |
| 224097_s_at | F11R | F11 receptor | 1.606 | 3.04E-07 |
| **Downregulated** | | | | |
| 336_at | TBXA2R | thromboxane A2 receptor | 2.794 | 9.29E-05 |
| 202806_at | DBN1 | drebrin 1 | 2.557 | 4.98E-10 |
| 205733_at | BLM | Bloom syndrome, RecQ helicase-like | 1.972 | 6.23E-12 |

## Appendix J: Genes recurrently up-regulated by lenalidomide in T cells

| Probe ID | Symbol | Name |
|---|---|---|
| **Upregulated** | | |
| 204567_s_at | ABCG1 | ATP-binding cassette, sub-family G (WHITE), member 1 |
| 219361_s_at | AEN | apoptosis enhancing nuclease |
| 224480_s_at | AGPAT9 | 1-acylglycerol-3-phosphate O-acyltransferase 9 |
| 229354_at | AHRR | aryl-hydrocarbon receptor repressor; programmed cell death 6 |
| 201951_at | ALCAM | activated leukocyte cell adhesion molecule |
| 201952_at | ALCAM | activated leukocyte cell adhesion molecule |
| 208950_s_at | ALDH7A1 | aldehyde dehydrogenase 7 family, member A1 |
| 227438_at | ALPK1 | alpha-kinase 1 |
| 201302_at | ANXA4 | annexin A4 |
| 201301_s_at | ANXA5 | annexin A4 |
| 204765_at | ARHGEF5 | Rho guanine nucleotide exchange factor (GEF) 5 |
| 235412_at | ARHGEF7 | Rho guanine nucleotide exchange factor (GEF) 7 |
| 1562270_at | ARHGEF8 | Rho guanine nucleotide exchange factor (GEF) 7 |
| 1562271_x_at | ARHGEF9 | Rho guanine nucleotide exchange factor (GEF) 7 |
| 202548_s_at | ARHGEF10 | Rho guanine nucleotide exchange factor (GEF) 7 |
| 1552691_at | ARL11 | ADP-ribosylation factor-like 11 |
| 218694_at | ARMCX1 | armadillo repeat containing, X-linked 1 |
| 225286_at | ARSD | arylsulfatase D |
| 219359_at | ATHL1 | ATH1, acid trehalase-like 1 (yeast) |
| 213238_at | ATP10D | ATPase, class V, type 10D |
| 220358_at | BATF3 | basic leucine zipper transcription factor, ATF-like 3 |
| 59644_at | BMP2K | BMP2 inducible kinase |
| 219546_at | BMP2K | BMP2 inducible kinase |
| 226853_at | BMP2K | BMP2 inducible kinase |
| 209920_at | BMPR2 | bone morphogenetic protein receptor, type II (serine/threonine kinase) |
| 210214_s_at | BMPR2 | bone morphogenetic protein receptor, type II (serine/threonine kinase) |
| 228937_at | C13ORF31 | chromosome 13 open reading frame 31 |
| 224574_at | C17ORF49 | chromosome 17 open reading frame 49 |
| 222883_at | C1ORF163 | chromosome 1 open reading frame 163 |
| 238365_s_at | C1ORF228 | chromosome 1 open reading frame 228 |
| 242520_s_at | C1ORF228 | chromosome 1 open reading frame 228 |
| 228067_at | C2ORF55 | chromosome 2 open reading frame 55 |
| 220770_s_at | C5ORF54 | chromosome 5 open reading frame 54 |
| 223276_at | C5ORF62 | MSTP150 |
| 229070_at | C6ORF105 | chromosome 6 open reading frame 105 |
| 230301_at | C7ORF46 | chromosome 7 open reading frame 46 |
| 209726_at | CA11 | carbonic anhydrase XI |
| 209031_at | CADM1 | cell adhesion molecule 1 |
| 209032_s_at | CADM1 | cell adhesion molecule 1 |
| 212765_at | CAMSAP1L1 | calmodulin regulated spectrin-associated protein 1-like 1 |
| 217196_s_at | CAMSAP1L1 | calmodulin regulated spectrin-associated protein 1-like 1 |
| 212763_at | CAMSAP1L1 | calmodulin regulated spectrin-associated protein 1-like 1 |
| 225045_at | CCDC88A | coiled-coil domain containing 88A |
| 216598_s_at | CCL2 | chemokine (C-C motif) ligand 2 |

| Probe ID | Symbol | Name |
|---|---|---|
| 201743_at | CD14 | CD14 molecule |
| 206508_at | CD70 | CD70 molecule |
| 225081_s_at | CDCA7L | cell division cycle associated 7-like |
| 202284_s_at | CDKN1A | cyclin-dependent kinase inhibitor 1A (p21, Cip1) |
| 206380_s_at | CFP | complement factor properdin |
| 223249_at | CLDN12 | claudin 12 |
| 208791_at | CLU | clusterin |
| 231499_s_at | CLU | clusterin |
| 227522_at | CMBL | carboxymethylenebutenolidase homolog (Pseudomonas) |
| 201438_at | COL6A3 | collagen, type VI, alpha 3 |
| 64486_at | CORO1B | coronin, actin binding protein, 1B |
| 214508_x_at | CREM | cAMP responsive element modulator |
| 230511_at | CREM | cAMP responsive element modulator |
| 209967_s_at | CREM | cAMP responsive element modulator |
| 207630_s_at | CREM | cAMP responsive element modulator |
| 221541_at | CRISPLD2 | cysteine-rich secretory protein LCCL domain containing 2 |
| 1555889_a_at | CRTAP | cartilage associated protein |
| 201380_at | CRTAP | cartilage associated protein |
| 1554464_a_at | CRTAP | cartilage associated protein |
| 210844_x_at | CTNNA1 | catenin (cadherin-associated protein), alpha 1, 102kDa |
| 200765_x_at | CTNNA1 | catenin (cadherin-associated protein), alpha 1, 102kDa |
| 200764_s_at | CTNNA1 | catenin (cadherin-associated protein), alpha 1, 102kDa |
| 200839_s_at | CTSB | cathepsin B |
| 208923_at | CYFIP1 | cytoplasmic FMR1 interacting protein 1 |
| 219179_at | DACT1 | dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis) |
| 218094_s_at | DBNDD2 | dysbindin (dystrobrevin binding protein 1) domain containing 2 |
| 203409_at | DDB2 | damage-specific DNA binding protein 2, 48kDa |
| 239310_at | DDB2 | damage-specific DNA binding protein 2, 48kDa |
| 200862_at | DHCR24 | 24-dehydrocholesterol reductase |
| 224374_s_at | EMILIN2 | elastin microfibril interfacer 2 |
| 207111_at | EMR1 | egf-like module containing, mucin-like, hormone receptor-like 1 |
| 201341_at | ENC1 | ectodermal-neural cortex (with BTB-like domain) |
| 201719_s_at | EPB41L2 | erythrocyte membrane protein band 4.1-like 2 |
| 213365_at | ERI2 | exoribonuclease 2 |
| 221664_s_at | F11R | F11 receptor |
| 224097_s_at | F11R | F11 receptor |
| 223000_s_at | F11R | F11 receptor |
| 238484_s_at | FADS2 | fatty acid desaturase 2 |
| 238861_at | FADS2 | fatty acid desaturase 2 |
| 213861_s_at | FAM119B | family with sequence similarity 119, member B |
| 221856_s_at | FAM63A | family with sequence similarity 63, member A |
| 219785_s_at | FBXO31 | F-box protein 31 |
| 227265_at | FGL2 | fibrinogen-like 2 |
| 204834_at | FGL2 | fibrinogen-like 2 |
| 235574_at | GBP4 | guanylate binding protein 4 |
| 235175_at | GBP4 | guanylate binding protein 4 |
| 223887_at | GPR132 | G protein-coupled receptor 132 |

| Probe ID | Symbol | Name |
|---|---|---|
| 228027_at | GPRASP2 | G protein-coupled receptor associated sorting protein 2 |
| 211284_s_at | GRN | granulin |
| 200678_x_at | GRN | granulin |
| 216041_x_at | GRN | granulin |
| 208886_at | H1F0 | H1 histone family, member 0 |
| 232397_at | HECW2 | HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 |
| 210387_at | HIST1H2BC | histone cluster 1 |
| 210387_at | HIST1H2BE | histone cluster 1 |
| 210387_at | HIST1H2BF | histone cluster 1 |
| 210387_at | HIST1H2BG | histone cluster 1 |
| 210387_at | HIST1H2BI | histone cluster 1 |
| 221606_s_at | HMGN5 | nucleosomal binding protein 1 |
| 219403_s_at | HPSE | heparanase |
| 222881_at | HPSE | heparanase |
| 229450_at | IFIT3 | interferon-induced protein with tetratricopeptide repeats 3 |
| 1565817_at | IKZF1 | IKAROS family zinc finger 1 (Ikaros) |
| 226759_at | IKZF4 | IKAROS family zinc finger 4 (Eos) |
| 226761_at | IKZF4 | IKAROS family zinc finger 4 (Eos) |
| 229752_at | IKZF4 | IKAROS family zinc finger 4 (Eos) |
| 205992_s_at | IL15 | interleukin 15 |
| 217371_s_at | IL15 | interleukin 15 |
| 204562_at | IRF4 | interferon regulatory factor 4 |
| 235182_at | ISM1 | isthmin 1 homolog (zebrafish) |
| 200922_at | KDELR1 | KDEL endoplasmic reticulum protein retention receptor 1 |
| 244370_at | KIAA2022 | KIAA2022 |
| 230636_s_at | KLF9 | Kruppel-like factor 9 |
| 221221_s_at | KLHL3 | kelch-like 3 (Drosophila) |
| 206486_at | LAG3 | lymphocyte-activation gene 3 |
| 202252_at | LOC100131294 | RAB13, member RAS oncogene family; similar to hCG24991 |
| 201951_at | LOC100133690 | activated leukocyte cell adhesion molecule |
| 201952_at | LOC100133690 | activated leukocyte cell adhesion molecule |
| 206600_s_at | LOC100133772 | similar to MCT; solute carrier family 16, member 5 |
| 213590_at | LOC100133772 | similar to MCT; solute carrier family 16, member 5 ( |
| 223095_at | LOC100270710 | hypothetical LOC100270710; MARVEL domain containing 1 |
| 236124_at | LOC153546 | hypothetical protein LOC153546 |
| 227415_at | LOC283508 | hypothetical protein LOC283508 |
| 240581_at | LOC644135 | hypothetical LOC644135 |
| 225745_at | LRP6 | low density lipoprotein receptor-related protein 6 |
| 226770_at | MAGI3 | membrane associated guanylate kinase, WW and PDZ domain containing 3 |
| 223095_at | MARVELD1 | hypothetical LOC100270710; MARVEL domain containing 1 |
| 218773_s_at | MSRB2 | methionine sulfoxide reductase B2 |
| 202944_at | NAGA | N-acetylgalactosaminidase, alpha- |
| 228073_at | NANP | N-acetylneuraminic acid phosphatase |
| 224802_at | NDFIP2 | Nedd4 family interacting protein 2 |
| 224799_at | NDFIP2 | Nedd4 family interacting protein 2 |
| 235759_at | NECAB1 | N-terminal EF-hand calcium binding protein 1 |
| 207075_at | NLRP3 | NLR family, pyrin domain containing 3 |

| Probe ID | Symbol | Name |
|---|---|---|
| 219557_s_at | NRIP3 | nuclear receptor interacting protein 3 |
| 223535_at | NUDT12 | nudix (nucleoside diphosphate linked moiety X)-type motif 12 |
| 218400_at | OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa |
| 209485_s_at | OSBPL1A | oxysterol binding protein-like 1A |
| 217890_s_at | PARVA | parvin, alpha |
| 1554256_a_at | PCNXL2 | pecanex-like 2 (Drosophila) |
| 229354_at | PDCD6 | aryl-hydrocarbon receptor repressor; programmed cell death 6 |
| 226731_at | PELO | pelota homolog (Drosophila) |
| 220952_s_at | PLEKHA5 | pleckstrin homology domain containing, family A member 5 |
| 204519_s_at | PLLP | plasma membrane proteolipid (plasmolipin) |
| 202446_s_at | PLSCR1 | phospholipid scramblase 1 |
| 241916_at | PLSCR1 | phospholipid scramblase 1 |
| 208890_s_at | PLXNB2 | plexin B2 |
| 209826_at | PPT2 | palmitoyl-protein thioesterase 2 |
| 208121_s_at | PTPRO | protein tyrosine phosphatase, receptor type, O |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase |
| 202252_at | RAB13 | RAB13, member RAS oncogene family; similar to hCG24991 |
| 219622_at | RAB20 | RAB20, member RAS oncogene family |
| 209568_s_at | RGL1 | ral guanine nucleotide dissociation stimulator-like 1 |
| 200885_at | RHOC | ras homolog gene family, member C |
| 223168_at | RHOU | ras homolog gene family, member U |
| 227983_at | RILPL2 | Rab interacting lysosomal protein-like 2 |
| 213397_x_at | RNASE4 | ribonuclease, RNase A family, 4 |
| 213566_at | RNASE6 | ribonuclease, RNase A family, k6 |
| 222573_s_at | SAV1 | salvador homolog 1 (Drosophila) |
| 218276_s_at | SAV1 | salvador homolog 1 (Drosophila) |
| 213716_s_at | SECTM1 | secreted and transmembrane 1 |
| 1560676_at | SIAH3 | seven in absentia homolog 3 (Drosophila) |
| 219386_s_at | SLAMF8 | SLAM family member 8 |
| 206600_s_at | SLC16A5 | similar to MCT; solute carrier family 16, member 5 |
| 213590_at | SLC16A5 | similar to MCT; solute carrier family 16, member 5 |
| 212810_s_at | SLC1A4 | solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 |
| 219869_s_at | SLC39A8 | solute carrier family 39 (zinc transporter), member 8 |
| 213113_s_at | SLC43A3 | solute carrier family 43, member 3 |
| 227620_at | SLC44A1 | solute carrier family 44, member 1 |
| 228485_s_at | SLC44A1 | solute carrier family 44, member 1 |
| 229898_at | SNX33 | sorting nexin 33 |
| 212558_at | SPRY1 | sprouty homolog 1, antagonist of FGF signaling (Drosophila) |
| 210829_s_at | SSBP2 | single-stranded DNA binding protein 2 |
| 203787_at | SSBP2 | single-stranded DNA binding protein 2 |
| 1552739_s_at | ST7L | suppression of tumorigenicity 7 like |
| 204068_at | STK3 | serine/threonine kinase 3 (STE20 homolog, yeast) |
| 203069_at | SV2A | synaptic vesicle glycoprotein 2A |
| 1565579_at | TATDN2 | TatD DNase domain containing 2 |
| 238067_at | TBC1D8B | TBC1 domain family, member 8B (with GRAM domain) |
| 211276_at | TCEAL2 | transcription elongation factor A (SII)-like 2 |
| 201666_at | TIMP1 | TIMP metallopeptidase inhibitor 1 |

| Probe ID | Symbol | Name |
|---|---|---|
| 202085_at | TJP2 | tight junction protein 2 (zona occludens 2) |
| 206271_at | TLR3 | toll-like receptor 3 |
| 1558102_at | TM6SF1 | transmembrane 6 superfamily member 1 |
| 234994_at | TMEM200A | transmembrane protein 200A |
| 207113_s_at | TNF | tumor necrosis factor (TNF superfamily, member 2) |
| 214228_x_at | TNFRSF4 | tumor necrosis factor receptor superfamily, member 4 |
| 207426_s_at | TNFSF4 | tumor necrosis factor (ligand) superfamily, member 4 |
| 241819_at | TNFSF8 | tumor necrosis factor (ligand) superfamily, member 8 |
| 203476_at | TPBG | trophoblast glycoprotein |
| 204083_s_at | TPM2 | tropomyosin 2 (beta) |
| 209129_at | TRIP6 | thyroid hormone receptor interactor 6 |
| 220775_s_at | UEVLD | UEV and lactate/malate dehyrogenase domains |
| 202893_at | UNC13B | unc-13 homolog B (C. elegans) |
| 205019_s_at | VIPR1 | vasoactive intestinal peptide receptor 1 |
| 204165_at | WASF1 | WAS protein family, member 1 |
| 217975_at | WBP5 | WW domain binding protein 5 |
| 1558586_at | ZNF33B | zinc finger protein 33B |
| 220661_s_at | ZNF692 | zinc finger protein 692 |
| 204026_s_at | ZWINT | ZW10 interactor |

## Appendix K: Genes recurrently down-regulated by lenalidomide in T cells

| Probe ID | Symbol | Name |
|---|---|---|
| **Downregulated** | | |
| 204671_s_at | ANKRD6 | ankyrin repeat domain 6 |
| 229215_at | ASCL2 | achaete-scute complex homolog 2 (Drosophila) |
| 205733_at | BLM | Bloom syndrome, RecQ helicase-like |
| 220560_at | C11ORF21 | chromosome 11 open reading frame 21 |
| 218641_at | C11ORF95 | hypothetical protein LOC65998 |
| 227806_at | C16ORF74 | chromosome 16 open reading frame 74 |
| 242509_at | C16ORF74 | chromosome 16 open reading frame 74 |
| 235063_at | C20ORF196 | chromosome 20 open reading frame 196 |
| 223623_at | C2ORF40 | chromosome 2 open reading frame 40 |
| 213268_at | CAMTA1 | calmodulin binding transcription activator 1 |
| 1555370_a_at | CAMTA1 | calmodulin binding transcription activator 1 |
| 1553102_a_at | CCDC69 | coiled-coil domain containing 69 |
| 201005_at | CD9 | CD9 molecule |
| 202806_at | DBN1 | drebrin 1 |
| 235378_at | FAM161B | family with sequence similarity 161, member B |
| 219709_x_at | FAM173A | family with sequence similarity 173, member A |
| 1555974_a_at | FLJ39051 | hypothetical protein LOC399972 |
| 1555973_at | FLJ39051 | hypothetical protein LOC399972 |
| 220131_at | FXYD7 | FXYD domain containing ion transport regulator 7 |
| 231771_at | GJB6 | gap junction protein, beta 6, 30kDa |
| 205929_at | GPA33 | glycoprotein A33 (transmembrane) |
| 212070_at | GPR56 | G protein-coupled receptor 56 |
| 212143_s_at | IGFBP3 | insulin-like growth factor binding protein 3 |
| 210095_s_at | IGFBP3 | insulin-like growth factor binding protein 3 |
| 227750_at | KALRN | kalirin, RhoGEF kinase |
| 244623_at | KCNQ5 | potassium voltage-gated channel, KQT-like subfamily, member 5 |
| 242517_at | KISS1R | KISS1 receptor |
| 236043_at | LOC100130175 | hypothetical protein LOC100130175 |
| 222307_at | LOC282997 | hypothetical protein LOC282997 |
| 205041_s_at | ORM1 | orosomucoid 1 |
| 210448_s_at | P2RX5 | purinergic receptor P2X, ligand-gated ion channel, 5 |
| 201876_at | PON2 | paraoxonase 2 |
| 228113_at | RAB37 | RAB37, member RAS oncogene family |
| 240690_at | SGK223 | homolog of rat pragma of Rnd2 |
| 207554_x_at | TBXA2R | thromboxane A2 receptor |
| 336_at | TBXA2R | thromboxane A2 receptor |
| 214183_s_at | TKTL1 | transketolase-like 1 |

## Appendix L: Impact of lenalidomide on the gene expression of CLL NK cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 232397_at | NA | NA | 4.092 | 3.59E-07 |
| 240581_at | LOC644135 | uncharacterized LOC644135 | 3.621 | 1.94E-13 |
| 213566_at | RNASE6 | ribonuclease, RNase A family, k6 | 3.133 | 4.84E-09 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 2.735 | 3.47E-08 |
| 225710_at | GNB4 | guanine nucleotide binding protein, beta 4 | 2.635 | 6.14E-10 |
| 223535_at | NUDT12 | nudix-type motif 12 | 2.393 | 2.04E-06 |
| 218400_at | OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa | 2.306 | 1.94E-07 |
| 228910_at | NA | NA | 2.269 | 3.85E-06 |
| 224374_s_at | EMILIN2 | elastin microfibril interfacer 2 | 2.260 | 1.01E-12 |
| 203153_at | IFIT1 | interferon-induced tetratricopeptide repeats 1 | 2.223 | 6.27E-07 |
| 207075_at | NLRP3 | NLR family, pyrin domain containing 3 | 2.183 | 2.34E-07 |
| 219386_s_at | SLAMF8 | SLAM family member 8 | 2.151 | 9.33E-07 |
| 228067_at | KIAA1211L | KIAA1211-like | 2.097 | 4.69E-07 |
| 220770_s_at | C5orf54 | chromosome 5 open reading frame 54 | 2.081 | 6.59E-12 |
| 204439_at | IFI44L | interferon-induced protein 44-like | 2.081 | 5.74E-04 |
| 242714_at | NA | NA | 2.075 | 4.69E-07 |
| 204466_s_at | SNCA | synuclein, alpha | 2.035 | 2.64E-05 |
| 208923_at | CYFIP1 | cytoplasmic FMR1 interacting protein 1 | 2.001 | 5.02E-08 |
| 230147_at | F2RL2 | coagulation factor II (thrombin) receptor-like 2 | 1.985 | 4.77E-03 |
| 223484_at | C15orf48 | chromosome 15 open reading frame 48 | 1.963 | 7.63E-03 |
| 211284_s_at | GRN | granulin | 1.930 | 2.74E-10 |
| 216041_x_at | GRN | granulin | 1.929 | 8.94E-10 |
| 207949_s_at | ICA1 | islet cell autoantigen 1, 69kDa | 1.918 | 1.16E-08 |
| 225532_at | CABLES1 | Cdk5 and Abl enzyme substrate 1 | 1.898 | 4.61E-05 |
| 200678_x_at | GRN | granulin | 1.843 | 6.14E-09 |
| 1565817_at | IKZF1 | IKAROS family zinc finger 1 (Ikaros) | 1.809 | 1.97E-05 |
| 212458_at | SPRED2 | sprouty-related, EVH1 domain containing 2 | 1.778 | 6.42E-06 |
| 204083_s_at | TPM2 | tropomyosin 2 (beta) | 1.778 | 1.32E-06 |
| 225207_at | PDK4 | pyruvate dehydrogenase kinase, isozyme 4 | 1.771 | 3.40E-03 |
| 210547_x_at | ICA1 | islet cell autoantigen 1, 69kDa | 1.760 | 5.98E-09 |
| 229450_at | IFIT3 | interferon-induced tetratricopeptide repeats 3 | 1.752 | 4.38E-06 |
| 205977_s_at | EPHA1 | EPH receptor A1 | 1.738 | 5.85E-05 |
| 221618_s_at | TAF9B | TAF9B RNA polymerase II | 1.706 | 1.65E-06 |
| 206978_at | CCR2 | chemokine (C-C motif) receptor 2 | 1.695 | 7.81E-04 |
| 204747_at | IFIT3 | interferon-induced tetratricopeptide repeats 3 | 1.693 | 6.92E-06 |
| 218694_at | ARMCX1 | armadillo repeat containing, X-linked 1 | 1.691 | 6.93E-06 |
| 240115_at | LOC100286925 | uncharacterized LOC100286925 | 1.669 | 1.33E-04 |
| 205552_s_at | OAS1 | 2'-5'-oligoadenylate synthetase 1, 40/46kDa | 1.647 | 4.43E-05 |
| 202252_at | RAB13 | RAB13, member RAS oncogene family | 1.623 | 2.90E-03 |
| 204438_at | MRC1 | mannose receptor, C type 1 | 1.611 | 2.77E-02 |
| 203904_x_at | CD82 | CD82 molecule | 1.610 | 3.68E-06 |
| 208636_at | ACTN1 | actinin, alpha 1 | 1.609 | 7.71E-04 |
| 223249_at | CLDN12 | claudin 12 | 1.606 | 4.58E-06 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| 213056_at | FRMD4B | FERM domain containing 4B | 1.596 | 9.40E-07 |
| **Downregulated** | | | | |
| 212187_x_at | PTGDS | prostaglandin D2 synthase 21kDa (brain) | 2.575 | 4.46E-06 |
| 211748_x_at | PTGDS | prostaglandin D2 synthase 21kDa (brain) | 2.443 | 1.18E-05 |
| 36499_at | CELSR2 | cadherin, EGF LAG seven-pass G-type receptor 2 | 2.043 | 1.21E-09 |
| 226548_at | SBK1 | SH3-binding domain kinase 1 | 1.967 | 1.94E-13 |
| 211663_x_at | PTGDS | prostaglandin D2 synthase 21kDa (brain) | 1.879 | 3.69E-04 |
| 230312_at | NA | NA | 1.793 | 2.20E-04 |
| 204684_at | NPTX1 | neuronal pentraxin I | 1.705 | 8.18E-09 |
| 226549_at | SBK1 | SH3-binding domain kinase 1 | 1.643 | 5.48E-11 |
| 229215_at | ASCL2 | achaete-scute complex homolog 2 (Drosophila) | 1.640 | 2.95E-09 |

## Appendix M: Impact of lenalidomide on the gene expression of healthy NK cells

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Upregulated** | | | | |
| 232397_at | NA | NA | 3.454 | 4.51E-06 |
| 213668_s_at | SOX4 | SRY (sex determining region Y)-box 4 | 3.212 | 8.50E-07 |
| 240581_at | LOC644135 | uncharacterized LOC644135 | 2.972 | 2.24E-11 |
| 239764_at | LOC100996539 | uncharacterized LOC100996539 | 2.439 | 4.25E-10 |
| 204466_s_at | SNCA | synuclein, alpha | 2.401 | 4.35E-06 |
| 201418_s_at | SOX4 | SRY (sex determining region Y)-box 4 | 2.400 | 6.73E-07 |
| 223366_at | ZNF704 | zinc finger protein 704 | 2.385 | 4.50E-06 |
| 238263_at | EPHA1-AS1 | EPHA1 antisense RNA 1 | 2.377 | 4.34E-07 |
| 201417_at | SOX4 | SRY (sex determining region Y)-box 4 | 2.291 | 7.56E-07 |
| 201416_at | SOX4 | SRY (sex determining region Y)-box 4 | 2.282 | 2.58E-06 |
| 205174_s_at | QPCT | glutaminyl-peptide cyclotransferase | 2.268 | 6.73E-07 |
| 228910_at | NA | NA | 2.267 | 5.24E-06 |
| 235079_at | ZNF704 | zinc finger protein 704 | 2.240 | 2.49E-06 |
| 229254_at | MFSD4 | major facilitator superfamily domain containing 4 | 2.191 | 1.31E-08 |
| 226213_at | ERBB3 | v-erb-b2 erythroblastic leukemia viral homolog 3 | 2.140 | 6.64E-10 |
| 225710_at | GNB4 | guanine nucleotide binding protein, beta 4 | 2.120 | 2.34E-08 |
| 228067_at | KIAA1211L | KIAA1211-like | 2.072 | 7.60E-07 |
| 205977_s_at | EPHA1 | EPH receptor A1 | 1.977 | 1.58E-05 |
| 207949_s_at | ICA1 | islet cell autoantigen 1, 69kDa | 1.963 | 1.42E-08 |
| 202018_s_at | LTF | lactotransferrin | 1.923 | 4.48E-04 |
| 210547_x_at | ICA1 | islet cell autoantigen 1, 69kDa | 1.866 | 4.93E-09 |
| 237597_at | NA | NA | 1.857 | 2.78E-04 |
| 204099_at | SMARCD3 | SWI/SNF, matrix, regulator of chromatin, D3 | 1.817 | 1.20E-06 |
| 208923_at | CYFIP1 | cytoplasmic FMR1 interacting protein 1 | 1.802 | 3.35E-07 |
| 204083_s_at | TPM2 | tropomyosin 2 (beta) | 1.795 | 1.67E-06 |
| 219753_at | STAG3 | stromal antigen 3 | 1.777 | 6.53E-06 |
| 222941_at | USP46 | ubiquitin specific peptidase 46 | 1.760 | 6.68E-07 |
| 213566_at | RNASE6 | ribonuclease, RNase A family, k6 | 1.759 | 1.73E-05 |
| 224374_s_at | EMILIN2 | elastin microfibril interfacer 2 | 1.744 | 2.70E-10 |
| 203940_s_at | VASH1 | vasohibin 1 | 1.720 | 2.96E-06 |
| 203904_x_at | CD82 | CD82 molecule | 1.715 | 2.34E-06 |
| 207075_at | NLRP3 | NLR family, pyrin domain containing 3 | 1.709 | 7.45E-06 |
| 1556932_at | NA | NA | 1.700 | 4.52E-06 |
| 224928_at | SETD7 | SET domain containing (lysine methyltransferase) 7 | 1.692 | 2.02E-07 |
| 1556423_at | VASH1 | vasohibin 1 | 1.688 | 3.16E-06 |
| 210367_s_at | PTGES | prostaglandin E synthase | 1.673 | 1.44E-06 |
| 206425_s_at | TRPC3 | transient receptor potential cation channel, C3 | 1.670 | 2.30E-05 |
| 204467_s_at | SNCA | synuclein, alpha | 1.659 | 3.75E-05 |
| 205479_s_at | PLAU | plasminogen activator, urokinase | 1.651 | 1.20E-03 |
| 238726_at | NA | NA | 1.607 | 6.73E-07 |
| 210814_at | TRPC3 | transient receptor potential cation channel, subfamily C, member 3 | 1.590 | 3.08E-04 |

| Probe ID | Symbol | Name | $Log_2$ FC | Adjusted p value |
|---|---|---|---|---|
| **Downregulated** | | | | |
| 219371_s_at | KLF2 | Kruppel-like factor 2 (lung) | 2.212 | 0.000 |
| 1552321_a_at | CCDC65 | coiled-coil domain containing 65 | 2.147 | 0.000 |
| 211748_x_at | PTGDS | prostaglandin D2 synthase 21kDa (brain) | 2.018 | 0.000 |
| 212187_x_at | PTGDS | prostaglandin D2 synthase 21kDa (brain) | 2.013 | 0.000 |
| 1552320_a_at | CCDC65 | coiled-coil domain containing 65 | 1.931 | 0.000 |
| 36499_at | CELSR2 | cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila) | 1.866 | 0.000 |
| 230312_at | NA | NA | 1.749 | 0.000 |
| 226863_at | FAM110C | family with sequence similarity 110, member C | 1.722 | 0.002 |

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